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Methods for Quantitative Bioassay of Ehrlich Ascites-Tumor Cells [1,2]

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The use of ascites tumors as a source of relatively homogeneous animal cells has received increased recognition as an aid to quantitative biological and biochemical experimentation (*1–5*). Accurate estimates of the proportion of viable cells in a suspension would increase the usefulness of animal cells for many types of research, in particular for studies of the effects of viruses on host cells. Ascites tumors provide optimal material for quantitative bioassay of animal cells, since easily counted and uniformly dispersed cell suspensions may be obtained without the use of procedures that might affect cellular viability.

Quantitative bioassay of tumor cells has been attempted by Shear (*6*), Reinhard *et al.* (*7*), Craigie (*8*), and Eckert *et al.* (*9*). For a bioassay method to be well suited for quantitative experiments, however, it is desirable that a relatively high degree of accuracy be obtained over a wide range of doses with a minimal number of animals. The systems employed by the above authors did not fully satisfy these criteria.

The assay methods that were investigated and will be described and evaluated are based on the relationship of the following variables to number of viable cells in the inoculum:

1) Gain in weight of mice inoculated intraperitoneally.
2) Time until development (latent period) of palpable tumors in mice inoculated subcutaneously.
3) Proportion of subcutaneous inoculations that resulted in tumor formation.
4) Proportion of mice killed following intracerebral inoculation.
5) Survival time of mice inoculated intracerebrally with doses producing 100 percent mortality.

The first 3 methods were found to have limited usefulness. The fourth method gave fair estimates, but the last one was found to be usable over

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the widest range of doses and was the most economical (precision per mouse) of the 5.

Materials and Methods

Mice.—Strain CF-1 mice, supplied by Carworth Farms, were used throughout this investigation. Initially only females weighing between 18 and 22 gm. were employed. During the course of the study, however, it was found that males were equally satisfactory.

Tumor.—The Ehrlich ascites tumor was received in 1952 from Dr. K. Sugiura of the Sloan-Kettering Institute for Cancer Research, New York, New York. Tumor cells were maintained routinely by intraperitoneal inoculation of 0.2 to 0.5 ml. of undiluted ascitic fluid at 6- to 8-day intervals. For washing, dilution, and *in vitro* maintenance of cells 2 balanced salt solutions were used: modified glucosol, a phosphate-buffered Tyrode-type solution, described by Fulton and Armitage (*10*), and herein referred to as glucosol, and PBS, a less strongly phosphate-buffered Tyrode-type solution without carbon source, described by Dulbecco (*11*). Cells were usually used for experiments 7 days after inoculation; suspensions containing more than 10 percent red cells were not used. Cell counts were done in hemocytometers, and enough chambers were filled so that at least 500 cells were counted for each determination. All cells larger than about 12μ in diameter were classed as tumor cells. This was shown to be justified by comparing differential counts made by this method and similar counts made from stained smears. These data were in agreement with the report of Patt and Blackford (*4*), who compared the 2 counting methods and also found them to be nearly identical. The use of size as a criterion of malignancy follows from the predominantly tetraploid chromosome number of the cells.

Inoculation techniques.—Intraperitoneal inoculations, usually in 0.5 to 1.0 ml. volumes, were made by introducing the needle subcutaneously and then puncturing the peritoneum about 1 cm. from the point of entry. Subcutaneous inoculations, in 0.05 ml. amounts, were done in 4 locations on each mouse: 2 at the anterior axillary line, bilaterally, and 2 over the lower quadrants of the abdomen, bilaterally.

Intracerebral inoculations, in 0.03 ml. amounts, were made in either of 2 locations: the sagittal suture at about the level of the coronal suture or through the squamosal bone on a line between the outer canthus of the left eye and the external auditory canal. One-quarter inch #27 needles were used. The latter route was technically easier and resulted in less operative mortality. Mice dying during the first 2 days following inoculation were arbitrarily considered to have died due to injury received in the course of inoculation and were therefore excluded from the data.

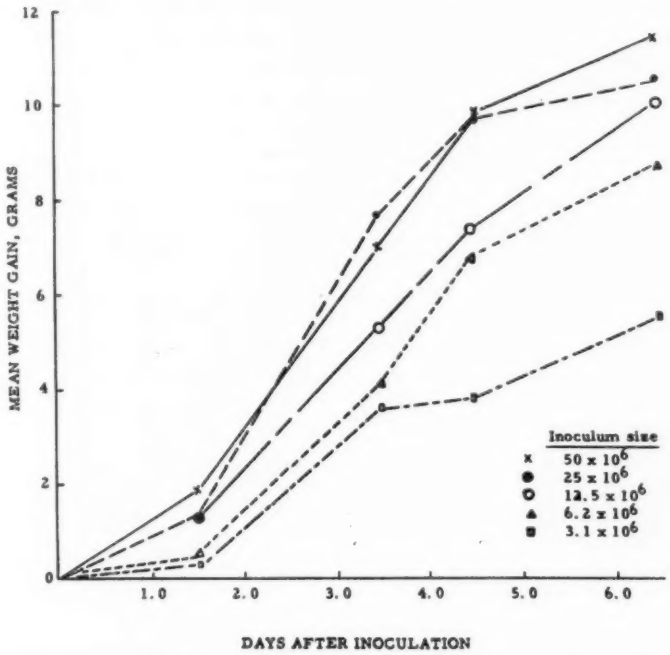
Results

Weight Gain as an Index of Number of Viable Cells Inoculated Intraperitoneally

Experiments were carried out to determine whether weight gain could be utilized as an index of the number of viable cells inoculated. Cells

were suspended in glucosol to a concentration of 5×10^7 cells per ml. Serial 2-fold dilutions of cells were prepared and 1.0 ml. of each dilution was inoculated intraperitoneally into each of 6 mice. Each animal was weighed at intervals for a week.

Text-figure 1 shows the effect of varied inocula on the mean weight gain of mice as a function of time. These data indicate that mean weight gain at any time between 3 and 6 days after inoculation can be used as an index of the number of viable cells inoculated—between approximately 25×10^6 and 3.1×10^6 cells per inoculum. The weight gain produced by the most concentrated inoculum employed (50×10^6) was not consistently larger than that produced by the first-diluted inoculum. Klein has shown that inocula containing less than approximately 800,000 cells irregularly produce a delayed ascites (*5*). Similar results have been obtained with the strain of tumor employed in these experiments. Such a level of inoculum would, therefore, be expected to be the lower limit of this technique. The use of weight gain as a method of bioassay is thus hampered by the limited range within which estimation is possible. For example, the largest inoculum which could be employed would be approximately 2.5×10^7 cells and the maximal decrease in concentration of viable cells which could be quantitatively determined would be approxi-



TEXT-FIGURE 1.—Effect of number of cells inoculated intraperitoneally on weight gain in mice. Mice were weighed individually. Each plotted point represents the arithmetic mean of 6 mice.

mately 25-fold. For this reason other quantitative bioassay methods were investigated.

Subcutaneous Inoculation for Titration of Viable Tumor Cells

It seemed possible that a combination of Reinhard's subcutaneous method of tumor-cell assay (*7*) and Bryan's latent-period determination, as applied to the assay of Rous sarcoma virus (*12*), might serve as a means for rapid tumor-cell assay. Table 1 summarizes the results of 3 experiments in which 4 aliquots of serial 3.2-fold ($10^{-0.5}$) dilutions were inoculated subcutaneously into each of 10 mice per dilution. Mice were examined at 24-hour intervals and the number of tumors palpable on each day was recorded.

Neither the mean latent periods for the development of palpable tumors (table 1) nor their reciprocals were linearly related with dose or log dose; furthermore, individual latent periods for a given dose were neither normally nor log-normally distributed. These findings are in part attributable to the relative inaccuracy of palpation as a method of latent-period determination when the latent period is short. There was no apparent transformation of the response for which this method of bioassay would have a practical statistical analysis.

Since the proportion of inoculations which result in tumor formation has been used as a method of bioassay for these cells (*13*), this measure was inspected. It will be noted (table 1) that all inocula containing more

TABLE 1.—*Effect of inoculum size on time of appearance and proportion of tumors arising after subcutaneous inoculation*

Expt.	Tumor cells per inoculum ($\times 10^4$)	Mean latent period* (*days*)	Maximum number tumors palpated†
1	800	2.0	40
	250	2.9	40
	80	3.0	40
	25	4.1	40
	8	‡	28
2	500	3.0	40
	160	3.0	40
	50	3.4	40
	16	5.2	40
	5	‡	34
3	500	2.2	40
	160	2.3	40
	50	3.2	40
	16	‡	35
	5	‡	4

*Arithmetic mean of periods elapsing between inoculation and palpation of tumors.

†Ten mice were inoculated in each of 4 loci—"maximum number of tumors palpated" takes into account the occasional regressions observed.

‡Mean latent period is not defined.

than 160,000 cells resulted in tumors and that the proportion of inocula producing tumors diminished rapidly with decreasing numbers of cells less than 160,000 per inoculum. If a linear relationship is assumed, it may be estimated, by extrapolation, that inocula containing fewer than approximately 10,000 cells would result in no tumors. Assays based on the proportion of subcutaneous inoculations resulting in tumors would therefore be useful only over a limited dose range.

In an attempt to increase the sensitivity of this assay, mice were irradiated with 390 r of whole-body X irradiation. These mice were still resistant to 20 times the dose necessary to cause tumor growth in 50 percent of mice inoculated by the intracerebral route. The attempt to employ the subcutaneous route for tumor-cell bioassay was therefore abandoned.

Tumor-Cell Bioassay by Intracerebral Route

A. LD50 determination.—In view of the high susceptibility of brain tissue to the growth of heterotransplants of tumor tissue (*14*), it was considered that an accurate assay for small numbers of viable Ehrlich ascites-carcinoma cells might be possible by intracerebral inoculation of tumor-cell suspensions.

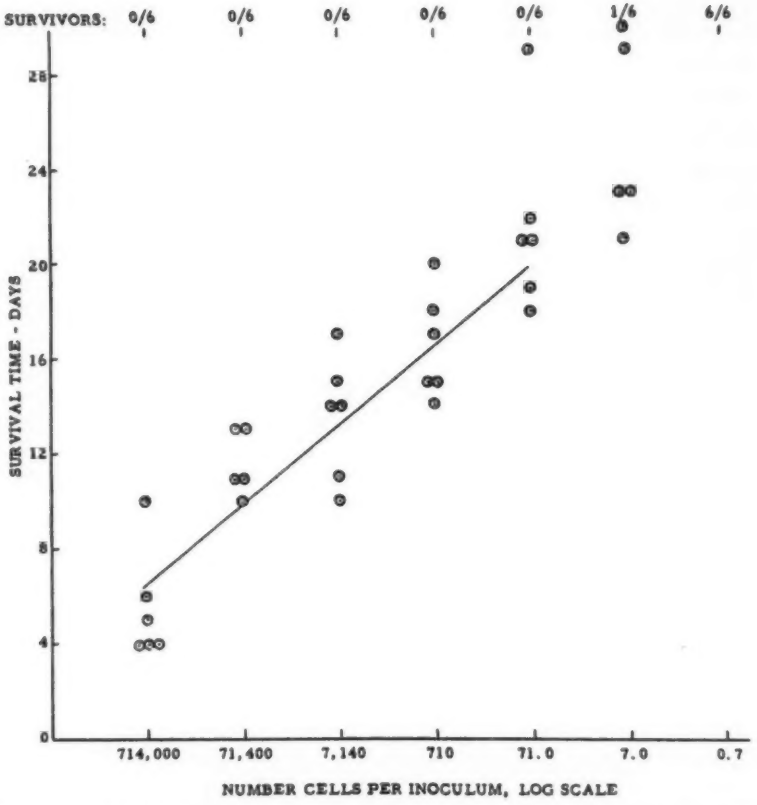
In order to determine the number of cells required to initiate tumor formation after intracerebral inoculation, groups of 6 mice each were inoculated via the "sagittal route" with serial dilutions of tumor cells suspended in PBS. The day on which inoculated mice died was recorded. The results of this experiment are presented in text-figure 2, which shows both the proportion of mice that survived each dilution and the survival times of individual mice that died. When estimated by the method of Reed and Muench (*15*), the data (proportion of survivors) revealed that the undiluted cell suspension contained $10^{5.49}$ LD50 doses. Since the cell suspension was estimated, by cell count, to contain $10^{5.85}$ cells ($\pm$ 20 percent),[4] it was calculated that 1 LD50 dose contained 2.3 cells.[5] Theoretically, if all cells were viable and independently capable of giving rise to a tumor, so that development of a tumor depends only on whether or not the inoculum contains at least 1 cell, and if the cells were distributed uniformly in the original suspension, then the LD50 unit should contain 0.693 cells, on the average.[6]

Since the data of the above experiment did not lend themselves well to Reed-Muench estimation, in order to investigate further the feasibility of this type of titration a series of experiments, similar to the above, was carried out. The results of these experiments are presented in table 2. Some of these experiments represent control titrations from experiments in which suspensions of unknown viability were being assayed. For each titration, at least 5 dilutions were employed; each dilution was inoculated

[4] Two standard error units.

[5] The standard error of this titration was estimated by the method of Pizzi (*16*) to be 0.32 on a log scale. The 95 percent confidence interval for the LD50 dose in this experiment is then 0.4 to 10 cells, which is approximately what one might expect from text-figure 2.

[6] When proportion of nonreactors = $0.5 = e^{-m}$, then $m = 0.693$ (Poisson formula).



TEXT-FIGURE 2.—Effect of number of cells inoculated intracerebrally on proportion of mice surviving and on survival time. Each point represents 1 mouse. Using the first 5 dilutions, the solid line is fitted to the points by the method of least squares, based on the knowledge of slope derived from a large number of experiments.

into 6 mice, and equal weights were arbitrarily assigned to the 11 estimates of the number of cells per LD50 unit; the average number of cells was 16. The ability to detect such a small number of cells in an inoculum indicated that the intracerebral route would allow assay over a wide dose range.

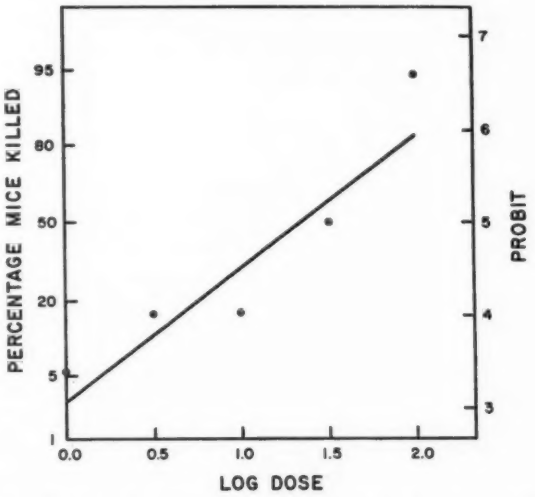
Probit analysis may be used instead of the Reed-Muench method to estimate the LD50 and its standard error. Such an analysis was carried out on the 3 essentially identical groups of experiments 1454-8 (table 2). Each group consisted of 30 mice, 6 mice being inoculated with each 3.2-fold dilution of cell suspension. Text-figure 3 shows the proportion of mice killed at each of the 5 doses and the computed probit regression line for the 3 groups combined.

The goodness of fit of the linear-regression line to these points was tested by the method described by Emmens (*17*). $\chi^2_2 = 5.07$, indicating that the probability of observing deviations from linear regression as

TABLE 2.—*Summary of intracerebral-tumor-cell titrations*

Expt.	Intracerebral route of inoculation	Medium for suspension of cells	Dilution interval	Estimated number of cells in LD50 unit
1453-P-2	Sagittal suture	PBS*	1:10	2.0
1453-P-3	" "	G† + P&S‡	1:10	7.7
1453-F-5	" "	PBS + P&S	1:3.2	38.0
1453-F-6	" "	PBS + P&S	1:10	10.0
1453-1-3	" "	G + P&S	1:3.2	38.0
1453-1-3	" "	G + NAF§	1:10	5.4
1454-1	" "	PBS + P&S	1:10	20.0
1454-8	Squamosal bone	G + 10% HS‖	1:3.2	10.0
1454-8	" "	G + 10% HS	1:3.2	15.0
1454-8	" "	G	1:3.2	15.0
1454-16-2	" "	G	1:10	15.0
Mean				16.0

*PBS = Dulbecco's phosphate-buffered saline.
†G = glucosol.
‡P&S = 100 units penicillin + 100 μg. streptomycin per ml.
§NAF = Normal allantoic fluid harvested from fertile chicken eggs incubated at 39° C. for 13 days.
‖G + 10% HS = 10 percent horse serum, inactivated for 30 minutes at 56° C. in glucosol.



TEXT-FIGURE 3.—Computed probit regression line relating log dose of Ehrlich ascites-tumor cells to percentage of mice killed after intracerebral inoculation with each dose. Each point represents the average of 3 experimental groups (from a single experiment), each containing 6 mice.

great, or greater, due to random sampling is between 0.10 and 0.05. It was thus not necessary to reject the hypothesis of linearity at the 0.05 level of probability.

The precision index (λ) originally proposed by Gaddum, and recently described by Bryan (*18*), may be employed to summarize the accuracy to

be expected from this titration. The precision index is the standard deviation (1 probit) divided by the slope of the probit regression line: 1 probit/1.461 probits per log dose = 0.685 log dose (*19*). The approximate minimal standard error, s, of the LD50 estimate, using 30 mice per titration, is given by the formula (*20*):

$$s = \frac{\lambda}{\sqrt{\frac{N^1}{2}}} = \frac{0.685}{\sqrt{15}} = 0.18$$

N^1 represents the number of animals in the significant response range, *e.g.*, 10 to 90 percent, and will therefore usually be less than the number of inoculated animals. The probit estimate of the standard error for this number of mice was 0.195 log number of cells. It should be emphasized that the use of 10-fold dilution increments, which is usually more practical if the LD50 cannot be estimated in advance, would reduce the accuracy of this titration due to the decrease in the proportion of inoculated animals that fall within the significant response range.

A relative disadvantage of the LD50 estimation as a bioassay method is that, in order to obtain an estimate with a standard error of 0.195 log number of cells, 30 mice are required within the significant response range. This would necessitate the inoculation of prohibitively large numbers, unless the LD50 could be accurately estimated in advance. The possibility that survival time might be employed as a measure of response in order to obtain increased precision with fewer animals was therefore investigated.

B. Survival-time method.—Since text-figure 2 suggested that a linear relationship existed between log dose and survival time, a number of experiments were carried out to ascertain whether survival time could be used as a satisfactory measure of response.

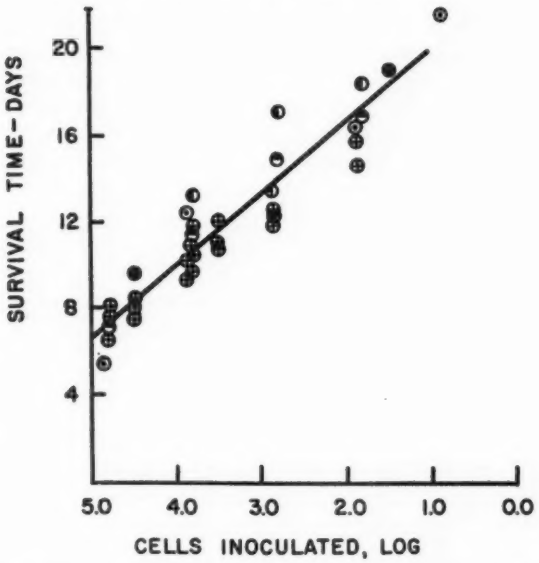
The following findings suggested that such would indeed be the case:

1) Time of death for a given dose was normally distributed.
2) The dose-response curve did not deviate significantly from linearity.
3) The estimates of the slopes of dose-response lines from many experiments were found not to differ significantly among themselves, their variation being no greater than that which could reasonably be expected due to chance.

The third finding permitted the calculation of a mean slope and the use of this estimate in the calculation of unknown assays. The above analyses, as well as the statistical evaluation of this bioassay method, are presented in the Appendix.

The combined results of 8 separate experiments, involving a total of 314 mice, are presented in text-figure 4. An interpretation of the possible implication of this curve will be given in the general discussion.

The linear relationship between log dose and survival time may be employed to yield information on the viability of any tumor-cell suspension as compared to a control untreated aliquot from the same original



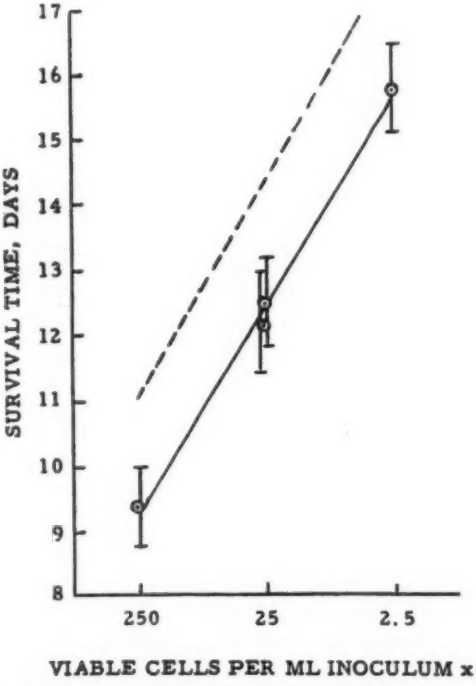
TEXT-FIGURE 4.—Effect of number of Ehrlich ascites-tumor cells inoculated on average survival time of mice after intracerebral inoculation. The line is fitted by the method of least squares, using experimental points weighted according to the number of mice per group. A total of 314 mice in 8 separate experiments contributed to this curve.

⊙ Experiment P-2, 6 mice per point.
◐ Experiment 16-1, 6 mice per point.
◓ Experiment 16-2, 6 mice per point.
● Experiment 16-3, 30 mice per point.
⊕ Miscellaneous 2-dilution control titrations, 6 to 10 mice per point.

source. This information may be expressed as a log potency ratio (difference in survival times of control and experimental groups divided by the slope of the dose-response line), or percent killing may be determined by simple calculation from the log potency ratio. Of particular interest, from the point of view of economy and expediency, is the fact that this assay may be carried out without any dilution of the unknown and control suspensions. It is applicable over the widest range (*i.e.*, a maximal change in number of viable cells can be quantitated) when no dilutions are made. It should be emphasized that in order to utilize this bioassay method without serial dilution of cells it is necessary to establish that the slope of the dose-response line does not change in the titration of a particular type of experimental group. A change would occur if the experimental treatment changed the growth rate of surviving cells.

For such a bioassay to be without systematic error it is also necessary that the presence of dead cells not affect the survival and growth rate of the viable cells in the suspension. Accordingly, an experiment was designed in which a cell suspension containing 2.5×10^7 cells per ml. was diluted 10^{-1}, 10^{-2}, and 10^{-3} in aliquots of a killed-cell suspension containing

2.5×10^7 cells per ml. The latter suspension had been heated at 56° C. for 15 minutes and did not produce tumors when inoculated undiluted into mouse brains. The results of this experiment are presented in text-figure 5. It will be seen that dilution in a suspension of killed cells did not affect the slope of the dose-response curve as compared to the control curve, based on results with cell suspensions containing similar numbers of tumor cells. If killed cells had an inhibitory action on the expression of viable cells, the dose-response curve would be expected to increase in slope. In other words, survival time would be expected to increase as the proportion of killed to viable cells rises. It is important to note that these data do not indicate the effect of injured but viable cells on the growth rate of uninjured cells in an Ehrlich ascites-tumor-cell suspension.



TEXT-FIGURE 5.—Effect of serial dilution of Ehrlich ascites-tumor cells in a suspension containing 2.5×10^7 heat-killed cells per ml. on the shape of the dose-response curve. *Vertical bars* indicate ± standard error of the mean. *Broken line* indicates the slope obtained from control experiments summarized in text-figure 4. This line has been displaced upward for clarity. *Each point* represents the mean survival time of 10 mice inoculated intracerebrally.

The precision index (*18*) may be employed to summarize the accuracy that might be expected from this assay. Using data from 34 groups of mice, 6 mice per group (2 groups had 30 mice and were therefore included 5 times each to give a weighted average), the mean standard deviation

was determined to be 1.90 days.[7] The precision index (λ) may then be calculated with the use of the mean slope (b) estimated from 17 experiments involving 310 mice:

$$\lambda = \frac{\sigma}{b} = \frac{s}{b} = \frac{1.90 \text{ days}}{3.33 \text{ days/log no. cells}} = 0.57 \text{ log no. cells.}$$

The total number (N) of mice required in an assay involving a control and an experimental group, in which a given accuracy ($2\ S.E._M = 0.5$) is required, may then be estimated according to the formula (*18*):

$$N = \frac{4\lambda^2}{(S.E._M)^2} = \frac{4(0.57)^2}{\left(\frac{0.5}{2}\right)^2} = 21.$$

This formula indicates that if a 95 percent confidence interval of the log potency ratio of 1.0 log unit in width is desired, a minimum of 11 mice per group is required. In order to confirm the above expectations in regard to the accuracy of the titration, it was necessary to carry out replicate determinations on a single cell suspension.

Therefore, an experiment was designed to determine the time available for replication without significant cell death. A cell suspension containing 2.0×10^7 cells per ml. was prepared in glucosol and placed at 0° C. in an ice bath. Immediately thereafter, and at hourly intervals for the next 6 hours, an aliquot was removed from the suspension and diluted 10-fold, after which 0.03 ml. was inoculated intracerebrally by the "squamosal route" into 6 mice per dilution. Two dilutions were used because at this time it was still desired to accumulate more information on the slope of the dose-response curve. The day of death of each mouse was recorded. The results of this experiment are presented in table 3. The variation in titers, presented as log potency ratios relative to the control, was no greater than would be expected by chance. Thus it was evident that there was no discernible death of cells at 0° C. over this time period. The results could therefore be used as a series of pure replications. Using the survival time averaged over both dilutions and weighted for the number of mice per group, the half width of the 95 percent confidence interval for individual titers = 0.363.[8] This compares well with the confidence intervals calculated from the individual determinations by the statistical methods described in the Appendix and listed in table 3. Confidence intervals of this magnitude are slightly, although not significantly, narrower than the 0.52 log dose (half width) predicted from the precision index.

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[7] $\bar{s}^2 = \frac{\Sigma s^2}{n} = \frac{152.03}{42} = 3.619.$

$\bar{s} = \sqrt{3.619} = 1.90$ days.

[8] $s_{\bar{d}}^2 = \frac{\Sigma d^2 - \frac{(\Sigma d)^2}{6}}{5} = 0.01990.$

$s_{\bar{d}} = \sqrt{0.01993} = 0.1411.$

95 percent confidence interval: $\bar{d} \pm 2.57 s_{\bar{d}} = 0.049 \pm 0.363 = -0.314$ to $+0.412$ log dose.

TABLE 3.—*Effect of time at 0° C. on viability of Ehrlich ascites-tumor cells*

Time cells held at 0° C. (*min.*)	Number of animals	Mean survival time* (*days*)	Potency ratio† (*log*)	S^2_d‡ (*log*)	95% Confidence interval§ (*log*)
0	12	9. 33			
60	12	9. 58	−0. 075	0. 031	0. 362
120	12	9. 25	+0. 024	0. 047	0. 451
190	11‖	9. 91	−0. 126	0. 048	0. 456
240	10‖	9. 10	+0. 069	0. 027	0. 343
300	11‖	8. 64	+0. 252	0. 050	0. 466
360	12	8. 83	+0. 150	0. 035	0. 389

*Six mice were inoculated for each of 2 dilutions, undiluted and 1:10; survival times were averaged over both dilutions.

†Log potency ratio relative to control = $\frac{\bar{x}_{\text{cont.}} - \bar{x}_{\text{unknown}}}{b} + (\bar{d}_c - \bar{d}_u)$, where b, the slope of the dose-response curve = 3.33 days per 10-fold decrease in concentration, and $\bar{d}$ equals dose of unknown (u) and control (c) groups.

‡S^2_d = variance of the log potency ratio. The method of calculation is given in the Appendix.

§Half width of 95 percent confidence interval of log potency ratio = $t \cdot s_d$ with $\alpha = 0.05$ and $N-1$ degrees of freedom.

‖Animals not included either escaped or died within 2 days after inoculation.

Discussion

It should be pointed out that each of the assay methods described in this report may have its place in different types of investigations. The intraperitoneal and subcutaneous inoculation methods readily detect experimental manipulations that kill the majority of tumor cells in a suspension. Experiments utilizing these routes may be designed to detect results such as: more than 90 percent of cells killed, or, in the case of the subcutaneous assay, more than 99 percent of cells killed. Such determinations require relatively small numbers of mice, are rapid, and hence are suitable for large-scale screening procedures. The intraperitoneal route has the advantage of rapidity, lack of weight gain of mice after a 7-day period being a suitable measure of more than 90 percent cell killing if an appropriate initial inoculum is used. The subcutaneous route can be employed to detect greater degrees of cell killing, if lack of tumor development is used as a criterion. The latter technique, however, requires a longer period of time for completion of the test.

The data presented indicate that the intracerebral inoculation methods are the most useful procedures for problems requiring more precise information relative to number of viable cells. In contrast to the methods outlined above, this route permits quantitative bioassays over a wide range of cell numbers. The intracerebral LD50 titration was found to be relatively less accurate per number of animals employed than the survival-time method of bioassay. Even if all animals fell into the significant response range, it would require 8 times as many animals as would be required by the latent-period method to achieve a given degree of accuracy in the determination of a log potency ratio. Furthermore, the LD50 assay method was more laborious than the latent-period assay, because it was necessary that several serial dilutions be made from each unknown suspension. The LD50-titration method does present the advantage that it is not necessary to assume the same growth rate for

the Ehrlich ascites-tumor cells which are viable after a given experimental procedure and control cells. It would be the method of choice if the growth rate were liable to change in a particular experiment. The survival-time method, having a high degree of accuracy (relative to other methods of tumor-cell bioassay) over a wide dose range, is the method of choice, however, for most quantitative investigations which require an estimate of the proportion of cells that are viable in a given suspension. This method is ideally suited to the quantitative determination of dose-response relationships.

It was shown that the mean number of cells per inoculum required to kill 50 percent of inoculated mice was of the order of 16. The interpretation of this finding depends in part on an accurate determination of the curve relating the proportion of mice dying with tumor to the concentration of cells inoculated. If this curve corresponded to a cumulative Poisson distribution, it would be likely that only a portion of cells were capable of giving rise to a tumor, whereas if this curve corresponded to a cumulative normal distribution, elements of host resistance could be implicated. Until these alternatives are resolved, the results of the LD50 titration must be expressed in terms of number of "LD50 units" rather than number of viable cells.

The finding of a linear relationship between log dose and survival time of mice inoculated intracerebrally would be compatible with the hypothesis that tumor cells grow at a constant exponential rate during the period when the curve is linear. The slope of this line would then directly reflect the growth rate of tumor cells in the mouse brain. Such an interpretation has been shown by Gard to be correct in the case of Theiler's GD VII virus inoculated into the mouse brain (*21*). This hypothesis, however, cannot be established for the data described in this report without direct determination of the intracerebral growth rate of Ehrlich ascites-tumor cells.

Summary and Conclusions

Weight gain of mice over a given period of time could be used as an index of the number of tumor cells inoculated intraperitoneally between approximately 2.5×10^7 and 1×10^6 cells per inoculum.

All subcutaneous inocula containing more than 160,000 cells resulted in tumor formation in mice. The time lapse until appearance of these tumors was not linearly related to the dose inoculated. The proportion of inocula resulting in tumors decreased as the number of cells in the inoculum decreased. It was estimated that inocula containing less than 10,000 cells would not give rise to progressively growing tumors.

Intracerebral inoculation of tumor cells into mice can be employed to determine the proportion of cells that are viable in a given suspension. The average number of cells per inoculum producing tumors in 50 percent of inoculated mice was estimated to be about 16 cells. The half width of the 95 percent confidence interval for the LD50 estimate, using 30 mice and 5 serial 3.2-fold dilutions, was about 0.5 on a log scale. If a potency

ratio between control and experimental groups is obtained with 30 mice per group, the half width of the 95 percent confidence interval of the log potency ratio would be increased by a factor of $\sqrt{2}$, *i.e.*, to 0.71.

The survival time of mice inoculated intracerebrally was found to be a linear function of the logarithm of the dose between about 30 and 500,000 cells per inoculum. The survival times of mice inoculated with a given dose followed a normal distribution. The slope of the dose-response line did not change over a variety of experiments. The expression of viable cells was not affected by the presence of heat-killed cells in the inoculum. From these findings it follows that bioassay of viable tumor cells, in terms of log potency ratios, may be carried out by inoculation of mice from a single aliquot of the treated cell suspension and from a single aliquot of the control suspension. Inoculating 12 mice with the treated suspension and 12 mice with the control suspension, the half width of the 95 percent confidence interval of the log potency ratio was estimated by the precision index to be 0.52. It is thus possible to obtain log potency ratios of a given degree of accuracy by this method with one eighth the number of animals required for such a determination by the LD50 method. By the use of this assay it was shown that Ehrlich ascites-tumor cells suspended in glucosol solution do not lose their viability when held for 6 hours at 0° C. Methods for the statistical analysis of this bioassay method are described in the Appendix.

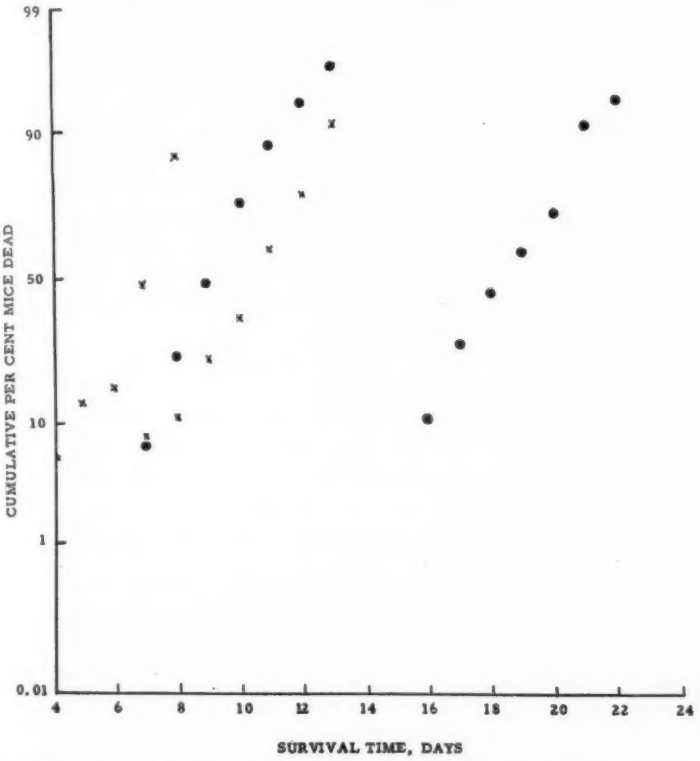
Appendix

This Appendix will summarize the experimental evidence which shows that the following 3 conditions hold sufficiently well to permit use of regression techniques in the statistical analysis of data obtained by the bioassay method of observing survival time of mice after intracerebral inoculation of Ehrlich ascites-tumor cells: (*a*) The relationship of survival time to the logarithm of the dose is linear over a wide range of doses. (*b*) Survival times of mice inoculated with any given dose follow a normal distribution. (*c*) The standard deviation of the survival times is independent of the dose. In addition, the Appendix will set forth the numerical evidence for several statements made in the text.

Normal Distribution of Survival Times

There were 4 large groups of mice in which all mice in a group were inoculated with the same dose. In the experiment which showed that there was no discernible loss of viability of cells held at 0° C. over a period of 6 hours (table 3), 39 mice were inoculated with 1 dose of cells and 41 mice were inoculated with a 10-fold dilution. In another experiment, 30

mice were inoculated with 1 dose of cells and 30 more mice were inoculated with a suspension of cells diluted 1,000-fold. The cumulative distributions of the survival times in the 4 groups are plotted on probability paper in text-figure A1. On probability paper one axis carries an arithmetic scale, but the scale on the other axis is transformed so that the cumulative percentage distribution of the normal distribution is a straight line with the slope inversely proportional to the standard deviation. For each of the 4 groups of mice the points lie sufficiently close to a straight line for the distribution of survival times to be regarded as normal.



TEXT-FIGURE A1.—Cumulative distributions of survival times for experimental groups, plotted on probability paper. There were 30 mice in each of the distributions represented by *dots*. There were 39 mice and 41 mice in the distributions represented by *crosses*.

Homogeneity of Variance

The 4 sets of points in text-figure A1 appear to be reasonably parallel, but the equality of standard deviations can best be tested by the variance ratio. The experiment with the 2 groups of 30 mice was designed to test the homogeneity of variance. More specifically, this experiment was designed to test the assumption that the variance (square of the standard

deviation) is independent of the dose against the alternative that for more dilute doses (and longer average survival times) the variance is larger. The ratio of the variance of the group inoculated with the more dilute dose to the variance of the group inoculated with the stronger dose is 1.51; the probability of observing a ratio of variances as great as or greater than 1.51 in 2 random samples of this size from normal universes with the same variance is greater than 0.10. Thus, it was concluded that the increase of variance with dilution, if any, is not great enough to preclude application of the analysis of covariance and normal-regression theory.

Linearity of Regression

Only experiments with more than 2 dose levels contain information about the linearity of the dose-response curve. Three such experiments were performed, 1 with 3 dose levels, 1 with 4, and 1 with 5. Following the customary test of linearity of regression, the sum of squared deviations of individual survival times from the linear-regression curve was compared with the sum of squared deviations within the groups, *i.e.*, the error sum of squares in analysis of variance. These comparisons are presented in table A1; in none of the 3 experiments would a polynomial of higher degree fit the data significantly better than a straight line. That is, in none of the 3 experiments were systematic departures from linearity of the dose-response curve evident.

TABLE A1.—*Linearity of regression—analysis of variance for data of 3 experiments*

Expt.	Soure of variation	Degrees of freedom	Sum of squares	Mean squares	Variance ratio (F)	Probability (P)
1453-P-2	Linear regression	1	792.1	792.1	102.9	<0.001
	Deviations from linear regression	3	46.5	15.5	2.01	>0.10
	Random sampling (error)	*25*	*193.3*	7.7		
	Total	29	1031.9			
1454-16-1	Linear regression	1	243.0	243.0	73.6	<0.001
	Deviations from linear regression	1	1.8	1.8	0.55	>0.40
	Random sampling (error)	*15*	*49.0*	3.3		
	Total	17	293.8			
1454-16-2	Linear regression	1	326.7	326.7	92.8	<0.001
	Deviations from linear regression	2	8.3	4.15	1.18	>0.20
	Random sampling (error)	*20*	*70.3*	3.52		
	Total	23	405.3			

Homogeneity of Slopes

There were 17 experimental units, involving 310 mice, from which independent estimates of the slope of the regression line could be obtained. Individual estimates of the slope varied from 2.27 to 5.17 days per 10-fold decrease in number of cells inoculated (table A2). It was important to

determine whether such variation of estimates indicates that the slope of the dose-response line changed from experiment to experiment or whether this variation is consistent with sampling variation in independent estimates of the same slope.

TABLE A2.—*Regression data from titrations*

Titration	Degrees of freedom	Sums of squares and products			Regression coefficient*	Errors of estimate	
		$\Sigma(x-\bar{x})^2$	$\Sigma x(d-\bar{d})$	$\Sigma(d-\bar{d})^2$		Sum of squares†	Degrees of freedom
1453-P-2	29	1031. 9	−218. 0	60. 0	−3. 63	239. 8	28
1454-16-1	17	293. 8	−54. 0	12. 0	−4. 50	50. 8	16
1454-16-2	23	405. 3	−99. 0	30. 0	−3. 30	78. 6	22
Control							
60 min.	11	62. 9	−11. 5	3. 0	−3. 83	18. 8	10
120 "	11	114. 2	−15. 5	3. 0	−5. 17	34. 1	10
190 "	10	61. 6	−8. 1	2. 73	−2. 97	37. 6	9
240 "	9	42. 9	−9. 5	2. 5	−3. 80	6. 8	8
300 "	10	52. 5	−6. 2	2. 73	−2. 27	38. 4	9
360 "	11	59. 7	−10. 0	3. 0	−3. 33	26. 4	10
1454-16-3	59	1516. 6	−421. 5	135. 0	−3. 12	200. 6	58
1454-29-1	11	62. 9	−9. 5	3. 0	−3. 17	32. 8	10
1454-25	11	64. 2	−10. 5	3. 0	−3. 50	27. 4	10
1454-32	11	42. 9	−8. 5	3. 0	−2. 83	18. 8	10
1454-35	17	97. 0	−14. 4	4. 44	−3. 25	50. 0	16
MG-1							
A-1	17	99. 0	−12. 6	4. 44	−2. 83	63. 4	16
MG-2	19	96. 8	−13. 5	5. 00	−2. 70	60. 3	18
A-2	17	112. 0	−14. 7	4. 44	−3. 30	63. 5	16
Total	293	4216. 2	−937. 0	281. 28		1048. 1	276
Average regression					−3. 331	1094. 9	292

*$\Sigma x(d-\bar{d})/\Sigma(d-\bar{d})^2$.
†$\Sigma(x-\bar{x})^2-[\Sigma x(d-\bar{d})]^2/\Sigma(d-\bar{d})^2$.

The sum of squared deviations of the survival times from 17 parallel lines is compared to the sum of squared deviations from 17 individual lines in Table A3. Since the latter sum is not significantly less than the former, *i.e.*, 17 individual lines do not describe the data significantly better than 17 parallel lines, the data are consistent with the assumption that the slope is constant.

TABLE A3.—*Analysis of errors of estimate from average regression*

Source of variation	Degrees of freedom	Errors of estimate		Variance ratio (F)	Probability (P)
		Sum of squares	Mean square		
Deviations from average regression (17 parallel lines)	292	1094. 9	3. 7497		
Deviations from individual titration regressions (17 individual lines)	276	1048. 1	3. 7975		
Differences among titration regressions	16	46. 8	2. 925	0. 77	>0. 50

Since there is no evidence that the slope of the dose-response curves varies (only the estimates of the slope vary), the combined estimate from all experiments has been used in the bioassay of unknown suspensions. The common slope of the 17 parallel lines determined by least squares is $b = 3.331$ days per 10-fold *decrease* in number of cells inoculated. The standard error of this estimate of the slope is:

$$S_b = \sqrt{\frac{\frac{1094.91}{292}}{281.3}} = \sqrt{\frac{3.75}{281.3}} = \sqrt{0.0133} = 0.115 \text{ days/log dose.}$$

Determination of Accuracy of Titration

The estimation of the potency of an unknown relative to a control is essentially the same when past experience is used to establish the slope of the dose-response line as when only current information is used. Geometrically, a line with a given slope is fitted by least squares to the points representing the control data and the sum of squared deviations of the points from this line is computed. A parallel line is fitted to the points representing the data from the suspension of unknown strength and the sum of squared deviations of the "unknown" points from this line is computed. The difference between the logarithm of the concentration of the unknown suspension and the logarithm of the concentration of the control, at their original dilutions (the log potency ratio), is estimated as the vertical distance between the 2 parallel lines divided by the slope, *i.e.*, the horizontal distance between the 2 lines:

$$M = \frac{\Delta}{b} = \frac{\bar{x}_c - \bar{x}_u}{b} + (\bar{d}_c - \bar{d}_u).$$

Where M is the log potency ratio:

Δ is the vertical distance between the lines:
b is the slope, 3.331 days/10-fold decrease in concentration;
$\bar{x}$ and $\bar{d}$ are the average survival time and average dose, respectively, for the mice inoculated with control and unknown suspensions.

The variance of this estimate is:

$$\text{Var } M = \frac{V_e}{b^2}\left(\frac{1}{n_c} + \frac{1}{n_u}\right) + \frac{\Delta^2 V_b}{b^4}$$

$$= 0.09013\ V_e\left(\frac{1}{n_c} + \frac{1}{n_u}\right) + 0.0001083\Delta^2$$

where V_e = the sum of the squared deviations of the points from their respective lines divided by $n_c + n_u - 2$;
n_c = number of mice inoculated with control suspensions;
n_u = number of mice inoculated with unknown suspensions;
V_b = variance of estimate of slope = 0.013331.

As an example of actual computations involved, consider the estimation of the log potency ratio and its 95 percent confidence interval for the

suspension held at 0° C. for 190 minutes (table 3). The complete data and some preliminary computations are presented in table A4.

$$V_e = \frac{10.33 + 15.00 + 3.68 + 35.24}{12 + 11 - 2} = \frac{64.25}{21} = 3.060$$

$$\Delta = 7.67 - 8.09 = -0.42 \text{ days}$$

$$M = \frac{-0.42}{+3.33} = -0.126 \text{ log dose}$$

$$\text{Var } M = 0.09013\ (3.060)\left(\frac{1}{12} + \frac{1}{11}\right) + 0.0001083\ (-0.42)^2$$

$$= 0.048055 + 0.000019 = 0.048074$$

$$S_M = \sqrt{0.048074} = 0.219 \text{ log dose}$$

95 percent confidence interval:

$M \pm 2.08\ (0.219) = -0.126 \pm 0.456$ log dose

2.08 is the 95 percent confidence interval coefficient from the t table with 21 degrees of freedom.

TABLE A4.—*Calculations involved in determination of confidence interval*

Material inoculated	Control—0 minutes		Unknown—190 minutes	
Log dose d	0	−1	0	−1
Survival times (*days*) $\bar{x}$	5	9	7	7
	7	10	8	9
	7	12	9	12
	7	12	9	12
	8	13	9	13
	9	13		14
Number	6	6	5	6
x (*days*)	7. 17	11. 50	8. 40	11. 17
$\Sigma(x-\bar{x})^2$	8. 83	13. 50	3. 20	34. 83
Mean $\bar{x}$ (*days*)	9.33		9.91	
$\bar{d}$ (*log dose*)	−0.5		−0.545	
Equation of line	$x = 9.33 - 3.33(d + 0.5)$		$x = 9.91 - 3.33(d + 0.545)$	
Expected* $\hat{x}$ (*days*)	7. 67	11. 0	8. 09	11. 43
Sum of sq.† dev. of points from line	10. 33	15. 0	3. 68	35. 24

* For example, $\hat{x} = 9.33 - 3.33(0 + 0.5) = 7.67$.

† $\Sigma(x - \bar{x})^2 + n(\bar{x} - \hat{x})^2$.

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Growth Curve of Rous Sarcoma Virus and Relationship of Infecting Dose to Yield of Virus in Chick Brain [1,2]

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Rous sarcoma virus (RSV) has been found to propagate in the brain of young chicks (*1–3*) and to kill in relation to dose (*3*). No gross lesions were observed in the brain, but a hemorrhagic area 0.5 to 10 mm. in diameter was almost invariably found in the meninges (*3*). Histologically, Rous sarcoma cells similar to those described by Vázquez-López (*1*) and by Duran-Reynals (*2*) were observed beneath the meninges but not invading the brain parenchyma (*4*). The data presented here show the growth curve of Rous sarcoma virus in chick brain and the relationship between the infecting dose of virus and the amount of virus recoverable from the brain.

Materials and Methods

Rous sarcoma virus (RSV), also commonly referred to as the chicken tumor I agent, was obtained from the National Cancer Institute, where it has been maintained in chickens by serial passage. Unsexed White Leghorn chicks, 3 to 7 days of age, known to be free from *Salmonella pullorum*, were used. Frozen, stable, standard virus was kindly provided by Dr. W. R. Bryan of the National Cancer Institute. Such standard virus was prepared by differential centrifugation from tumor tissue (*5*) and stored in a dry-ice chest at −50 to −70° C.

Inoculation of chicks.—Chicks were placed under light ether anesthesia and inoculated intracerebrally into the right hemisphere with 0.05 ml. of inoculum by means of a 1/8-inch 26-gauge needle. All chicks were observed daily for a period of 4 weeks and each chick was examined at the time of death for the presence of the typical hemorrhagic lesion in the meninges at the site of inoculation. Subcutaneous inoculation into the wing web was carried out as follows: Two-tenths ml. amounts were injected subcutaneously into the wing web with a 3/4-inch 26-gauge hypodermic needle. The needle was inserted through the muscle into the subcutaneous tissue of the wing web, thus avoiding leakage. Only the left wing was inoculated and the birds were examined daily for a period

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of 3 weeks. Multiple typical tumors appeared 4 or more days after inoculation, depending upon the potency of the inoculum.

Analysis of data.—The reciprocal of time, in days, times 100 (*i.e.*, 100/days) has been found to be a suitable transformation for analysis of both time to death following intracerebral inoculation of virus (*3*) and latent period for tumor production in the wing following subcutaneous inoculation of virus (*6–8*). Dose-response data were analyzed by the graphic methods recently illustrated in detail for tumor latent periods (*6–8*) and time-to-death responses (*3*). The potencies of all tissue suspensions are expressed as percentages relative to the observed potency of the standard, which was taken to be 100 percent in each assay. This procedure is essentially the same as that described by Bryan *et al.* for determining the potency of unknown tumor suspensions by tumor latent period (*6–8*). Differences due to fluctuation in the level of sensitivity of various test lots of chicks [*see* recent review by Bryan (*9,10*)] were therefore eliminated through reference to a standard virus within each test.

Storage of tissue and preparation of tissue extracts.—Brains were removed only from living or moribund chicks. Dead birds were routinely discarded. Each brain to be assayed was divided in half, and each half was placed in a separate ampoule, which was then sealed in an oxygen-gas flame. All such tissue was stored in the dry-ice chest at −70° C. until assayed. This procedure was carried out as follows: First, the brain was removed and the cerebrum was separated from the cerebellum; next, the cerebrum was divided in half transversely, so that a portion of both hemispheres was included in each half; finally, the cerebellum was divided in half longitudinally. In this manner 2 identical pools of brain tissue were made available for assay. On the day of the test the tissues were thawed and ground in a mortar with sand, and sufficient saline (containing 2% by volume of inactivated normal horse serum) was added to make a final concentration of 10 percent brain tissue by weight. This suspension was then clarified by centrifugation at 3000 r.p.m. for 20 minutes. The total time between removal of the ampoule from the dry-ice chest and the inoculation of the last chick did not exceed 2 hours.

Results

Histopathology of the brain.—It will be recalled that no gross lesions were observed in the brain, although a hemorrhagic area was almost invariably observed in the meninges in chicks dead or dying after intracerebral inoculation of RSV (*3*). Eyestone (*4*) examined histologically the brain of 1 moribund chick and observed an abundance of Rous sarcoma cells beneath the meninges but not invading the brain parenchyma. Brains were removed from 3 moribund chicks in our laboratory, and histologic examination readily confirmed the histologic picture just described. After examination of the sections, it was estimated that the number of tumor cells adherent to the brain after removal from the chick was less than 5 percent of the total weight of the brain. This observation, together with the

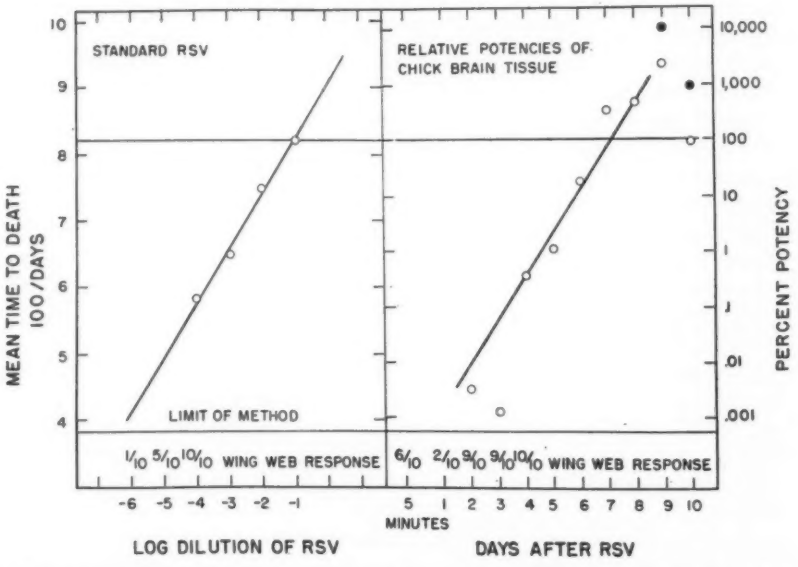
fact that subcutaneous tumors at the site of inoculation were never observed when standard RSV was employed and that RSV has been recovered in substantial quantities from nontumorous tissue, such as the chorioallantois (*11*) and liver (*12*) of infected embryonated eggs and from grossly unaltered liver of chickens inoculated intramuscularly with RSV (*13*), suggests that virus is propagating in the brain itself. However, the possibility cannot be excluded that the few tumor cells adherent to the brain after its removal may contain unexpectedly large amounts of RSV.

Growth curve of Rous sarcoma virus in chick brain.—Text-figure 1 shows the growth curve of Rous sarcoma virus in the brains of newly hatched chicks. The data on which this growth curve is based were derived from 974 chicks. The experiment consisted of 3 parts. First, 175 chicks were inoculated intracerebrally with standard Rous sarcoma virus diluted 1:10. Beginning 5 minutes after inoculation of virus, and daily thereafter, 3 chicks were killed each day and their brains removed and stored at −70° C. in sealed glass ampoules. When the birds began to die as a result of inoculation of virus, brain tissue was collected each day from both living and moribund birds. Dead birds were discarded. Second, the first aliquot (*see* Materials and Methods) of pooled brain tissue was inoculated into the wing web of young chicks. One aliquot of each of the frozen pools of brain tissue was thawed, ground in a mortar with sand, and suspended in saline to make a 10 percent suspension by weight. The suspension was clarified by centrifugation at 3000 r.p.m. for 20 minutes. Two-tenths ml. amounts of each suspension were then inoculated subcutaneously into the wing web of groups of 10 chicks each. The birds were examined daily for a period of 14 days and the number of chicks that developed tumors in the wing was recorded. At the same time, serial decimal dilutions of standard RSV were similarly inoculated into groups of 10 chicks each. These data are summarized at the bottom of text-figure 1. The third part of this experiment was conducted as follows: The remaining aliquots of brain tissue were thawed and ground in a mortar with sand, and sufficient saline was added to make a 10 percent suspension by weight. Each of these suspensions was then clarified by centrifugation at 3000 r.p.m. for 20 minutes. Five-hundredths ml. amounts of each suspension were inoculated intracerebrally into groups of 41 chicks each. At the same time, serial decimal dilutions of standard RSV were similarly inoculated into groups of 41 chicks each. All birds were examined daily for a period of 4 weeks.

At the conclusion of the experiment, the time to death for 50 percent of the chicks was estimated graphically for each group on probability paper [3] by plotting cumulative mortality against time as 100/days. As anticipated, the mean time to death of chicks inoculated with standard RSV was related linearly to the dose of virus. A potency of 100 percent was arbitrarily assigned to standard RSV diluted 1:10. Thus, it was possible to estimate graphically the relative potencies of the various suspensions of brain tissue. For example, the relative potency of brain tissue collected

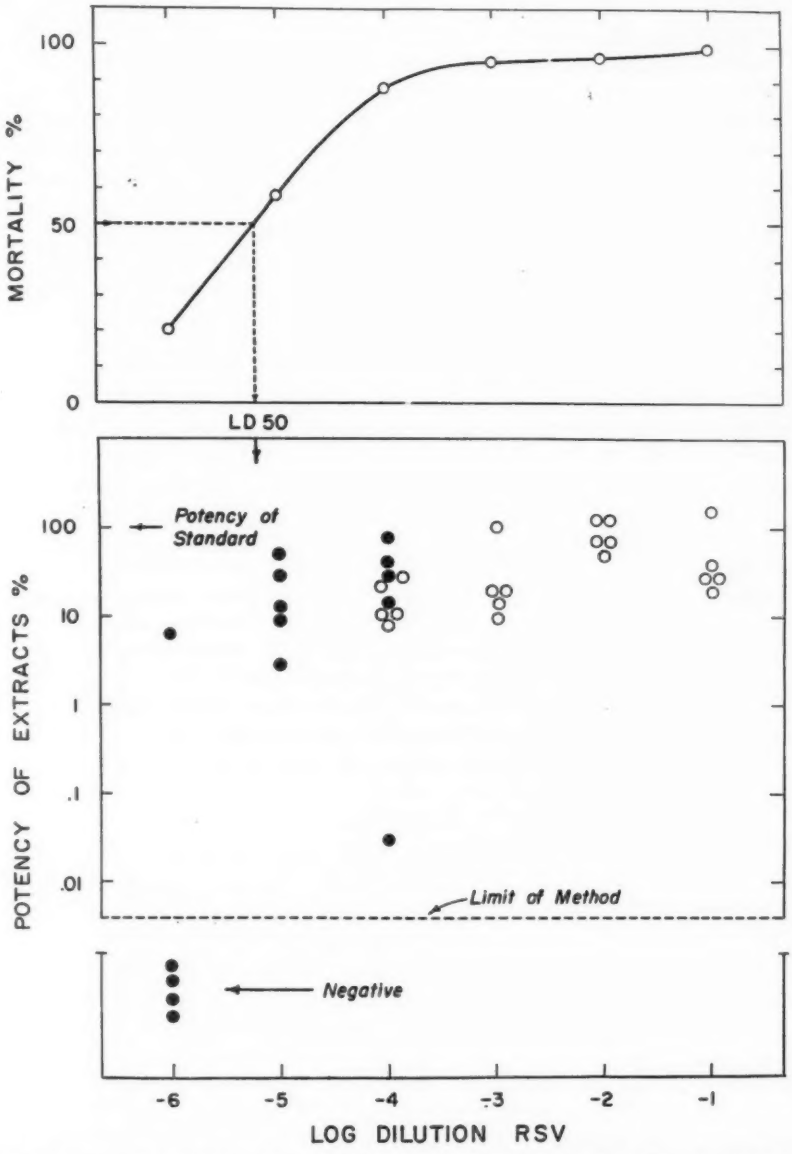
[3] No. 32,451 (normal ruling); Codex Book Co., Norwood, Mass.

on day 4 was estimated as follows: The mean time to death of the chicks inoculated intracerebrally with a 10 percent brain suspension was estimated graphically to be 6.3, expressed as 100/days, and the relative potency was then estimated graphically to be 0.14 percent. The limit of sensitivity of the method for any given experiment was determined by the susceptibility of that particular lot of chicks to RSV. Examination of the data summarized in text-figure 1 reveals: (*a*) Five minutes after inoculation of virus only 6 of 10 chicks inoculated into the wing with brain suspension developed tumors, thus indicating that infective virus disappeared very rapidly from the brain. (*b*) Very little virus could be recovered from brain tissue through the 3rd day after inoculation of RSV. (*c*) However, beginning with the 4th day after inoculation virus was present in relatively large amounts and increased at a regular rate until it reached its peak in moribund or prostrate birds. (*d*) The peak of viral potency attained was comparable to, or higher than, the standard virus used to initiate the infection. (*e*) Brain tissue removed from moribund birds was more potent in 2 instances than brain tissue removed on the same day from birds that appeared healthy. Utilizing the portion of the growth curve that showed a logarithmic increase in potency (*i.e.*, between the 4th and 9th days after inoculation), it can be calculated that during this period the time to double the potency of brain tissue was approximately 9.4 hours. Since the stability of RSV in brain tissue is not known, it is impossible to estimate what might be comparable to generation time in bacteria.



TEXT-FIGURE 1.—Growth curve of Rous sarcoma virus (RSV) in chick brain. ○ = Tissue collected from living chicks; ● = tissue collected from moribund chicks.

Effect of initiating dose on yield of virus.—Text-figure 2 summarizes data illustrating the relationship between the infecting dose of RSV and the amount of virus recoverable from the brain. The data collected in these



TEXT-FIGURE 2.—Relationship of infecting dose of RSV to yield of virus from chick brain. ○ = Experiment 1; ● = experiment 2.

experiments were based on 1,435 chicks. The experiment consisted of 2 parts. The first is shown in the upper portion of text-figure 2. Groups of 35 chicks each were inoculated intracerebrally with serial decimal dilutions of Rous sarcoma virus, as indicated. In addition to recording mortality in each of the groups, brains were removed from 5 moribund or prostrate chicks in each dilution group. Dead chicks were discarded. Each brain was placed individually in an ampoule that was then sealed in an oxygen-gas flame and stored at −70° C. The LD50 of standard Rous sarcoma virus was approximately $10^{-5.2}$. The second part of the experiment is shown in the lower portion of text-figure 2. The individual brains frozen from the first experiment were thawed and ground in a mortar with sand; then sufficient saline was added to make a 10 percent suspension by weight, which was clarified by centrifugation at 3000 r.p.m. for 20 minutes. Each of these suspensions was inoculated intracerebrally as a 10 percent suspension into groups of 35 chicks each. At the same time, 4 serial decimal dilutions of frozen standard RSV were inoculated intracerebrally into groups of 35 chicks each. The mean time to death of the various groups of chicks was estimated graphically as before and the relative potencies of the individual brains were estimated as illustrated in text-figure 1. These potencies have been plotted in the lower part of text-figure 2 against the dilution of RSV inoculated. It should be emphasized that each brain assayed was removed from a chick that was prostrate or moribund and showed the typical pathognomonic hemorrhagic area in the meninges at the site of inoculation. Examination of the data summarized in text-figure 2 reveals: (*a*) When chicks were inoculated intracerebrally with more than 1 LD50 of RSV, the brains contained appreciable and relatively uniform amounts of virus. (*b*) However, when chicks were inoculated with less than 1 LD50 of RSV, the brains contained, in 4 of 5 cases, no recoverable virus. These findings agree with and confirm those of Bryan *et al.*, who showed that when the initiating dose of Rous sarcoma virus was less than 1 ED50 the virus extractable from a given wing tumor was either very low or could not be detected at all (*14*).

Discussion

Taken as a whole, the growth curve of Rous sarcoma virus in chick brain parallels that of other animal viruses. Thus, 3 phases commonly observed with other viruses are also found with Rous sarcoma virus. These are: (*a*) A large proportion of the infective virus rapidly disappears from the tissue; then (*b*) a stationary phase or latent period occurs during which the infective-virus content of the tissue remains low, followed by (*c*) a progressive increase in viral concentration in the tissue. It appears that the growth curve of RSV in chick brain shows no obvious dissimilarity to that of the non-neoplastic animal viruses. Unfortunately, experimental data showing the relationship between the number of virus particles and the minimal infective dose are lacking. Consequently, little can be said regarding the stationary phase and its relationship to the so-called "eclipse phase" observed with bacteriophage and certain other viruses (*15*).

One of the most provocative observations in recent years was made by Bryan, Calnan, and Moloney (*14*), who found that the amount of virus extractable from a given tumor was directly related to the amount of virus used to initiate that tumor. Indeed, when high dilutions of virus were used, which produced tumors in less than half the chicks, 24 percent of such low-dose tumors yielded no extractable virus. The data presented in our report confirm this observation in chick brain. Failure to recover any infective virus from the brains of 4 of 5 moribund chicks inoculated with less than 1 LD50 of RSV is of considerable interest, particularly in view of the fact that the growth curve of RSV in chick brain clearly indicates that the maximum quantity of RSV is found in brain tissue removed from chicks just about to die. These data are also at variance with common experiences with many of the non-neoplastic animal viruses. In many laboratories, virus has been routinely recovered from dead or diseased animals inoculated with limiting dilutions of virus in order to establish the cause of death or disease. Although speculation concerning the nature of this phenomenon is premature at this time, it is of interest to recall the studies with the electron microscope made by Epstein (*16*), who observed that, at most, only 1 ascitic Rous sarcoma cell in 50 contained virus-like particles. Whether or not the infective-virus content of a given cell or tissue fluctuates with time is unknown.

Summary

The growth curve of Rous sarcoma virus (RSV) in chick brain paralleled that of many non-neoplastic animal viruses. First, virus disappeared rapidly from the brain, and 5 minutes after intracerebral inoculation of RSV, virus was barely detectable in brain tissue. This was followed by a stationary phase of approximately 3 days during which the infective-virus content of the brain remained low. Finally, beginning with the 4th day after inoculation, the viral content of the brain increased logarithmically and reached its maximum in moribund chicks. When chicks were inoculated intracerebrally with 1 to 10,000 lethal doses (LD50) of RSV and brains were removed only from moribund chicks, such brain tissue contained appreciable and relatively uniform amounts of virus. However, when chicks were inoculated intracerebrally with less than 1 LD50 of RSV, brains removed from the relatively few chicks that became moribund contained, in 4 of 5 instances, no recoverable RSV. This observation is in agreement with data on the yield of recoverable virus from tumor tissue [*see* Bryan, Calnan, and Moloney (*14*)] and is at variance with common experiences with many non-neoplastic viruses.

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Inhibition of Rous Sarcoma Virus Activity by Oxidized Tumor Lipides [1]

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One of the major difficulties encountered in studies on the purification of the Rous sarcoma virus is its labile nature. This lability is due, in part, to the lipide content of the virus or of the microsome fraction with which it is usually identified. After exhaustive extraction of this particulate fraction with organic solvents the lipide content has been estimated to be as high as 53 percent of the total dry-weight substance (*1*).

Claude (*2*) extracted from a relatively crude Rous tumor microsome preparation a lipide fraction that had the properties of a lecithin. He described this fraction as giving a strongly positive Schiff test for aldehydes and suggested the possibility that this chemically active group, which can be uncovered easily, plays a part in the spontaneous inactivation of the tumor agent. Glavind and Hartman (*3*) and, more recently, Kenaston *et al.* (*4*) showed that the thiobarbituric acid (TBA) test of Kohn and Liversedge (*5*) parallels the Kreis test for aldehydes. Since the thiobarbituric acid condenses with the oxidation products of unsaturated fatty acids to produce a compound with a characteristic light-absorption spectrum, this reaction may be used as a measure of the degree of oxidation of fatty acids, whether free or combined as phospholipides.

Wilbur *et al.* (*6*) have shown that oxidized unsaturated fatty-acid esters inhibit cell division. Employing the TBA reaction, Ottolenghi *et al.* (*7*) and Bernheim *et al.* (*8*) have inhibited certain mitochondrial-enzyme activities with oxidized methyl linolenate and oxidized methyl linoleate; Shuster (*9*) has demonstrated that respiration and glycolysis in Ehrlich ascites tumor are inhibited by the oxidation products of unsaturated fatty-acid esters; and Fisher and Wilbur (*10*) have found that deoxyribonucleic acid (DNA) is depolymerized by oxidized methyl linolenate.

Investigations in this laboratory have confirmed the extreme sensitivity of the Rous sarcoma virus to the more common organic solvents, in which there is complete loss of tumor-producing activity of the virus.

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[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

It has also been observed that pelletized virus materials will rapidly turn brown upon exposure to air, with a concomitant loss of potency. This browning is typical of lecithin during oxidation. The protection afforded the Rous sarcoma virus by either rabbit or horse serum and the stabilizing effect of citrate salts as opposed to phosphate salts have been reported previously (*11,12*). These are factors which have also been shown either to markedly inhibit the oxidation of unsaturated fatty acids (*13*), as measured by the TBA reaction, or, as in the case of phosphate buffer, to serve as an excellent medium for the oxidation process.

With this information available, the study presented herewith was undertaken in an effort to correlate the presence of oxidized unsaturated fatty acids in the microsome fraction of the Rous sarcoma with reduced biological activity. The demonstration of such an association would contribute to the understanding of at least one of the factors concerned with the lability or spontaneous inactivation of the virus particle.

Materials and Methods

Buffers.—Phosphate and citrate buffers (0.05 *M*, *p*H 6.0) were used as specified. Phosphate buffer was prepared from 0.1 *M* stock solutions of its salts (Na_2HPO_4–KH_2PO_4). Citrate buffer was prepared from 1.0 *M* stock salt solutions ($Na_3C_6H5O_7{\cdot}2H_2O$–$H_3C_6H5O_7{\cdot}H_2O$). Desired *p*H values were obtained by the appropriate combination of equimolar solutions of the members of the buffer pairs.

The buffer used as the diluent in the bioassay was 0.05 *M* citrate at *p*H 6.7, which contained 1.0 mg. of hyaluronidase[4] per 100 ml. of the diluent.

Virus materials.—A partially purified microsome fraction, standard virus preparation CT–632 (*14*) was used as the "substrate" in all tests designed to measure the effect of the fatty-acid oxidation products on biological activity. This frozen preparation was also used as the control or reference material for the quantitative potency estimates in all bioassays, with the exception of 1 experiment. In this case the reference material was a crude microsome fraction which was prepared, preserved, and designated standard virus preparation CT-581 (*15*).

The techniques for obtaining these virus materials from the Rous sarcoma tissue are briefly summarized as follows: The crude virus suspension (CT-581) was prepared by treating tumor tissue with hyaluronidase in 0.15 *M* potassium citrate. The microsome fraction was sedimented from the homogenate, which had been clarified 3 times, by centrifugation at 18,000 × g for 1 hour. The pellets were then resuspended and clarified twice, and the final supernatant, designated the crude microsome fraction, was recovered. The partially purified fraction (CT–632) was obtained by precipitation of the crude microsome fraction with protamine sulfate, elution of the virus from the protamine complex by trypsin treatment, and concentration of the material by differential centifugation. This fraction is referred to as a partially purified microsome or virus preparation.

[4] Hyaluronidase bovine testes, Worthington Biochemical Sales Co., Freehold, N. J.

The final concentration of the materials was 1.0 gm. equiv. per ml. (*i.e.*, 1.0 ml. of suspension for every gm. of tumor tissue processed). Aliquots of these suspensions were found to be stable when stored at −50 to −76° C. in 0.05 *M* citrate buffer, *p*H 6.7 Quantitative biological experiments have shown no significant variation among subsamples of a given virus preparation (*14,15*).

Microsome lipides.—Lipides were prepared by repeated petroleum-ether extraction of a freshly prepared crude microsome fraction obtained in a manner identical to that described for CT-581.

Lecithin was prepared from Rous sarcoma tissue according to the method described by Snider and Bloor (*16*) for the extraction of liver lecithin. The tissue was extracted with 95 percent ethanol and then filtered to remove the precipitated protein material. The alcohol filtrate was distilled under reduced pressure and the phospholipide extracted with petroleum ether. Repeated precipitation of the lecithin, from the ether extracts, with acetone, produced a clean, white preparation. Sphingomyelin was removed by centrifugation of the ether extracts.

It is possible that the final material contained trace amounts of cephalin.

Linolenic acid as the methyl ester was obtained from both the Hormel Foundation [5] and Mann Laboratories.[6]

Oxidation of the lipide materials was carried out by ultraviolet irradiation. The ultraviolet source was a Hanovia utility model quartz mercury arc lamp without filter.[7]

The materials were irradiated either at room termperature (25–28° C.) or at a temperature of 37° C. Room temperature was maintained by playing a stream of air onto the containers, and the higher temperature was controlled by means of a water bath. The containers were 150 ml. glass beakers partially covered with aluminum foil. In all cases the depth of the materials was less than 10 mm. and the distance to the ultraviolet source was 20 cm.

The thiobarbituric acid (TBA) reaction of Kohn and Liversedge as modified by Wilbur *et al.* (*17*) was used to determine the degree of lipide oxidation. The procedure was as follows: To 2.0 ml. of the oxidized samples (or 1.0 ml. diluted to 2.0 ml. with 0.05 *M* phosphate buffer, *p*H 6.0) was added 1.0 ml. of 20 percent trichloroacetic acid and 2.0 ml. of a 0.67 percent TBA solution (*5*). Color was developed for 10 minutes in a boiling water bath. When necessary, precipitates were removed by centrifugation at 4000 r.p.m. for 10 minutes in an ordinary centrifuge. The supernatants were read, against suitable blanks, at 530 mμ in a Coleman spectrophotometer (model #14).

Biological tests and analysis of data.—Appropriately diluted test or reference virus materials in 0.2 ml. amounts were inoculated subcutaneously into the wing web of 3-week-old chickens. The animals were of the New

[5] Hormel Foundation, Austin, Minn.

[6] Mann Laboratories, Inc., New York 6, N. Y.

[7] Hanovia Chemical and Mfg. Co., Newark, N. J. The electrical and spectral characteristics of the lamp are as follows: The tube operates on 105–125 volts and draws approximately 100 watts. The ultraviolet intensity of radiations of 3130 Å and shorter, measured at a distance of 20 inches, is over 250 μw. per cm.^{2}.

Hampshire Red strain and had been maintained on a standard diet in this laboratory from 1 day of age.

Interpretation of the biological responses and assays of virus activity were carried out by procedures previously described and illustrated in detail (*18,19*).

Procedures and Results

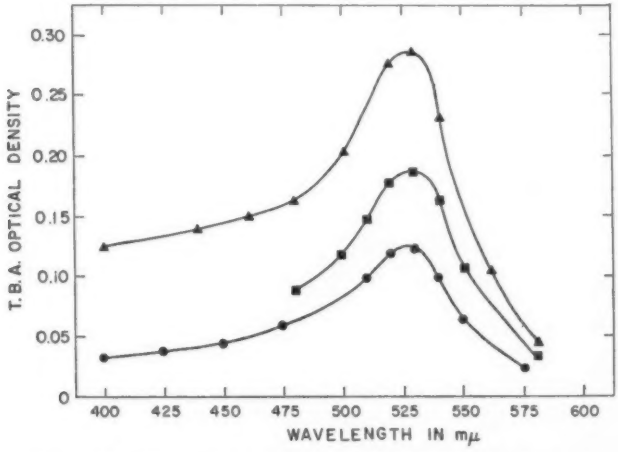
Chemical Studies

Demonstration of Oxidizable Lipides in Tissue Fractions of Rous Sarcoma by Use of Thiobarbituric Acid Test

The oxidation of unsaturated fatty acids, as measured by the TBA reaction, is catalyzed by ascorbic acid and accelerated by ultraviolet light (*13,17*). To demonstrate the presence of ultraviolet-sensitive lipides in partially purified and crude microsome fractions of the Rous sarcoma, the following experiments were carried out.

Lipides of the partially purified microsome fraction.—A freshly prepared, partially purified microsome fraction (*see* Materials and Methods) in citrate buffer was concentrated to two thirds its original volume by high-speed centrifugation (40,000 × g) for 60 minutes. The pelletized material was resuspended in 0.05 *M* *p*H 6.0 phosphate buffer and the suspended fraction was irradiated for 16 minutes at 37° C. At the end of this time a 2.0 ml. aliquot was sampled for the TBA reaction. The absorption spectrum of the chromogen produced is shown by the closed circles (●) in text-figure 1.

Lipides of the crude microsome fraction.—TBA chromogen production was identified with the lipide component of a crude microsome preparation by irradiation of the ether-soluble fraction and condensation of the oxida-



TEXT-FIGURE 1.—TBA absorption curves of oxidized lipides in Rous sarcoma tissue fractions. (▲) Rous tumor lecithin. (■) Crude microsome fraction. (●) Partially purified microsome fraction. (*See* text.)

tion products with the TBA reagent. In this experiment a freshly prepared crude microsome fraction (*see* Materials and Methods) was first concentrated by centrifugation for 1 hour at 40,000 × g. The sedimented material was then resuspended in a minimal amount of triple-distilled water to which was added 3 volumes of petroleum ether. The lipides were extracted by continuous shaking for 30 minutes and the ether phase was then irradiated at room temperature for 60 minutes. An aqueous solution of the oxidized fatty acids[8] was prepared by extraction of the irradiated sample for 20 minutes with triple-distilled water. To recover the aqueous phase free of ether droplets it was necessary to subject the emulsion to relatively low centrifugal force (2300 × g) for 10 minutes and to reduced pressure (20 inches Hg) for approximately 40 minutes. One ml. of the water extract was sampled for the TBA reaction. The absorption curve of the condensation product is denoted by the solid squares (■) in text-figure 1.

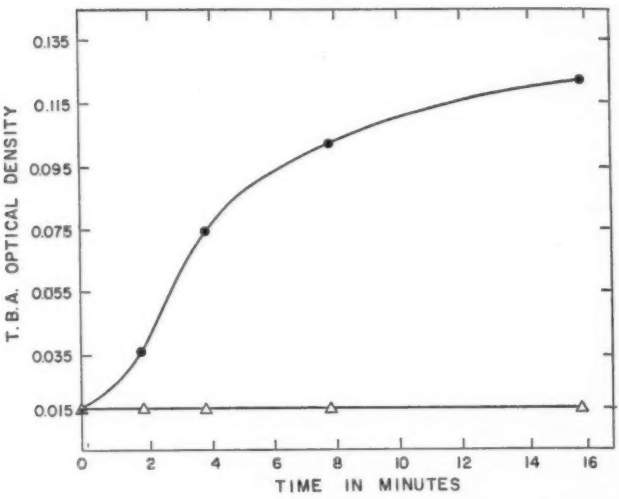
Lecithin of the Rous tumor tissue.—It seemed likely that the characteristic spectra for the compounds described above were due to the presence of the lecithin associated with the microsome fractions. To determine the correctness of this interpretation, lecithin extracted from the Rous tumor tissue was emulsified in 0.05 *M* phosphate buffer, *p*H 6.0 (in a concentration of 1 mg. per ml.), and irradiated for 250 minutes at 37° C. The TBA absorption spectrum obtained through the reaction of the oxidation products with the TBA reagent is illustrated by the solid triangles (▲) in text-figure 1.

It can be seen readily that the 3 curves have identical characteristics. The differences in the absorption maxima at 530 mμ are due to the relative degree of oxidation and concentration of the oxidation products in the materials. It should also be pointed out that the curves do not necessarily reflect complete extraction or quantitative measurement of the oxygen-labile lipides in the fractions. It is estimated, however, that the concentration of the oxidation products formed, under the conditions described, is of the order of micrograms [*see also* (*9*)].

The curves shown in the text-figure identify oxidizable lipides in the microsome fractions of the Rous tumor. They are characteristic and identical in form to those published by Bernheim *et al.* for oxidized lecithin (*21*) and Wilbur *et al.* for oxidized unsaturated fatty acids (*17*), as determined by the TBA reaction.

Relationship of time of irradiation to oxidation of the microsome lipides.—It is significant that oxidation of the microsome lipides, as measured by TBA chromogen production, is a function of time of irradiation. To demonstrate this relationship, an additional experiment was carried out in which a concentrated, partially purified microsome fraction prepared in 0.05 *M* phosphate buffer, *p*H 6.0, was irradiated at 37° C., and 2.0 ml. aliquots were sampled for the TBA reaction at intervals over a period of 16 minutes. As can be seen in text-figure 2, under these conditions nearly maximum lipide oxidation (as measured by chromogen production) is

[8] Bloor (*20*) reported that unsaturated fatty acids, when oxidized, become water-soluble, forming a clear solution.



TEXT-FIGURE 2.—Relationship of time of irradiation to oxidation of the lipides of a partially purified microsome fraction, as measured by TBA chromogen production. (●) Irradiated microsome suspension. (△) Control microsome suspension.

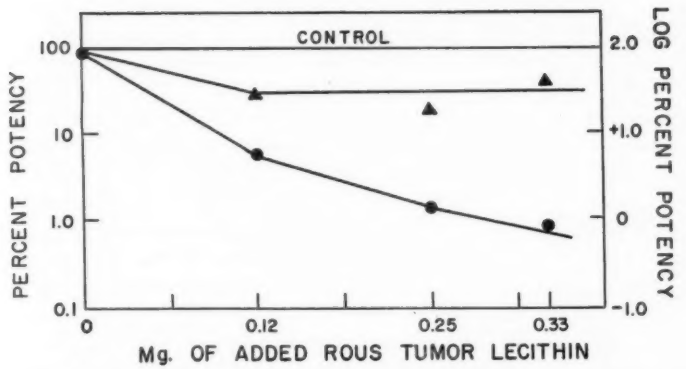
obtained after 16 minutes of irradiation. Within the same period, the control suspension, treated in an identical manner with the exception that it was completely shielded from the ultraviolet radiations, showed no increase in chromogen production. Results of this type can also be obtained with virus suspensions incubated for prolonged periods of time (up to 3 hours) in phosphate buffer at 37° C. in the presence of ascorbic acid.

Similar time-chromogen production relationships have been reported for oxidized lipides of mitochondria as well as lecithin and linolenic acid (*7,17*).

Biological Studies

Effect of Oxidized Lipides on Rous Sarcoma Virus Activity (Tumor-Producing Activity)

Attempts to show a direct correlation between degree of lipide oxidation in a dilute virus suspension and its biological activity (tumor-producing activity), by either ultraviolet irradiation or incubation of the microsome fraction with ascorbic acid, were unsuccessful with standard laboratory procedures. Ultraviolet light, employed under the conditions described herein, inactivates partially purified virus material in less than 2 minutes of irradiation. This is due, in part, to derangement of the bond structure of the nitrogenous bases associated with the nucleic acid component of the virus. Similarly, control virus suspensions incubated for prolonged periods of time in phosphate buffer are unstable and their bioassays are not reproducible (*12*). However, if virus lability or inactivation is due to the presence of oxidized lipides, then this effect could be duplicated by



TEXT-FIGURE 3.—Effect of Rous tumor lecithin on a partially purified Rous sarcoma virus preparation. (●) Oxidized lecithin. (▲) Unoxidized (control) lecithin.

the addition of these products to biologically active standard virus preparations.

Virus inhibition by oxidized Rous tumor lecithin.—In the first of 2 experiments designed to demonstrate this effect, Rous tumor lecithin was emulsified in 0.05 *M* phosphate buffer, *p*H 6.0, in a concentration of 1.0 mg. per ml. and divided into 2 equal lots. One lot was irradiated at 37° C. for approximately 2 hours[9] and the other, shielded from the radiations, was kept at room temperature to serve as a control emulsion. Serial dilutions of each lot were made and 0.5 ml. of each dilution was added to 4.5 ml. of standard virus preparation CT-632 (*14*), which had been diluted 1:100 with 0.05 *M* citrate buffer, *p*H 6.7. After incubation of all materials at 37° C. for 75 minutes they were sampled and diluted for bioassay. Each of the incubated samples was compared with a freshly thawed and diluted sample of reference virus suspension CT-632, the potency of which was taken to be 100 percent [for details of analysis and estimates of standard errors *see* (*18,19*)]. The results are illustrated in text-figure 3.

The abscissa indicates the amount, in milligrams, of Rous tumor lecithin added to the virus samples. The TBA optical-density value for the highest concentration of added lecithin was 0.041, and for the same amount of unoxidized lecithin the TBA value was 0.011. The latter value was due to rapid autoxidation of the lecithin upon exposure to air. It can be seen that lecithin, a normal component of tissue, when oxidized under the conditions described and added to an active virus suspension, reduced biological activity as much as 99 percent relative to the untreated virus suspension (indicated by the horizontal line at the 100-percent-potency level). In relation to the control (unoxidized lecithin) suspension, virus activity was inhibited more than 70 percent.

The second experiment of this group is a somewhat extended confirmation of the work of Kuck (*22*), who showed that citric acid "markedly"

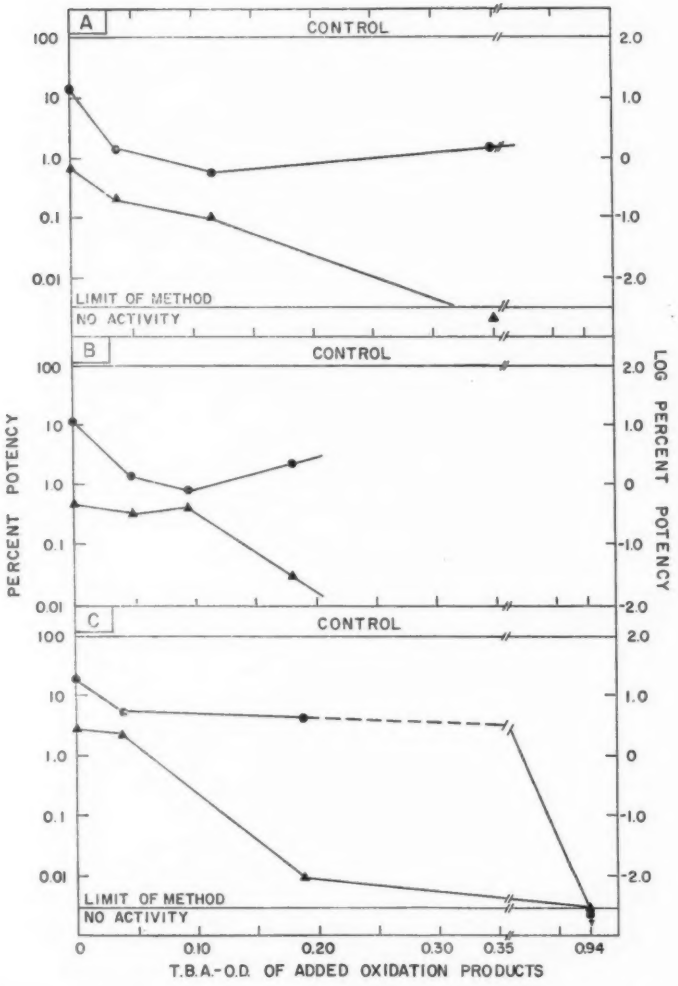
[9] Quantitative biological tests and qualitative chemical tests indicated there were no peroxides or other virus-inhibiting substances formed when phosphate buffer alone was irradiated under identical conditions.

inhibited the oxidation of liver fatty acids, as measured by the TBA reaction. In this study aqueous extracts of the oxidation products were used, thus preventing any oil-water interface reaction that might have had an indirect effect on the virus. Approximately 140 mg. of Rous tumor lecithin was taken up in 5.0 ml. of petroleum ether and irradiated at room temperature for 90 minutes. The water-soluble oxidation products were extracted as described above. Suitable dilutions of the aqueous extract were prepared and sampled for the TBA reaction; 0.5 ml. of each dilution was also added to 2.0 ml. of each of 2 virus substrate preparations, which were subsamples of standard virus preparation CT-632 diluted 10^{-2} with either 0.05 *M* *p*H 6.0 citrate buffer or 0.05 *M* *p*H 6.0 phosphate buffer. The materials were incubated at 37° C. for 75 minutes and then diluted and assayed for residual biological activity, as previously described (*19*). The results, as shown in part A of text-figure 4, indicate a direct relationship between amount of added oxidation products and potency of the virus suspensions. The reduction in biological activity of the test materials, at zero concentration of added oxidation products (in relation to the control virus material), was due to the effect of incubation on the dilute virus suspensions.

The effect of the water-soluble oxidation products of lecithin on the biological activity of Rous virus suspended in phosphate buffer is readily apparent. The tumor-producing activity was completely inhibited by the highest concentration of the oxidation products, whereas the citrate suspension, although somewhat reduced in relation to the control, due possibly to oxidation of the lipides associated with the treated virus, was still a highly active preparation.

Virus inhibition by oxidized microsome lipides.—In text-figure 1 it was seen that the TBA absorption curve of a water suspension of the oxidation products of the lipides (phospholipides with the properties of a lecithin) in a crude microsome fraction had the same characteristics as the spectrum presented by oxidized Rous tumor lecithin. The virus particle is associated with the microsome fraction, and there are indications that the particle itself consists, at least in part, of a lipide, perhaps complexed as a lipoprotein. Thus, in studies of active-virus recovery it would be of importance to understand the effect of the oxidized total-lipide component of a tumor microsome fraction on the Rous sarcoma virus.

This effect was demonstrated as follows: A crude virus suspension (crude microsome fraction) was concentrated by high-speed centrifugation. The sedimented particulate mass was resuspended in triple-distilled water and the lipide fraction recovered by repeated extraction with petroleum ether. Oxidation of the lipide extract was effected by ultraviolet irradiation for 90 minutes at room temperature. The oxidation products were recovered as a water extract, sampled for the TBA reaction, and diluted and added to the substrate virus suspensions as above (standard virus CT-632 in both citrate and phosphate buffers, 0.05 *M*, *p*H 6.0). To 4.0 ml. of a 10^{-2} dilution of the virus was added 1.0 ml. of the oxidized lipide extract. The test materials as well as suitable controls were incu-



TEXT-FIGURE 4.—Effect of aqueous extracts of oxidized lipides on biological activity of the Rous sarcoma virus (*see* text).

A. Effect of oxidized Rous tumor lecithin on (●) virus in citrate buffer; (▲) virus in phosphate buffer.

B. Effect of oxidized microsome lipides on (●) virus in citrate buffer; (▲) virus in phosphate buffer.

C. Effect of oxidized methyl linolenate on (●) virus in citrate buffer; (▲) virus in phosphate buffer.

bated for 75 minutes at 37° C. The materials were then sampled and diluted for bioassay. The control virus suspension used for bioassay was CT–581 (*see* Materials and Methods). This preparation, the potency of which was taken to be 100 percent, had been prepared in a manner

identical to that described above for the crude microsome fraction used as the lipide source.

Part B of text-figure 4 shows the relationship of added oxidized microsome lipides, in terms of TBA optical density, to residual potency of treated virus CT-632. The degree of inactivation of the virus in phosphate buffer at the highest concentration of the added oxidized microsome lipide is the same (within error of the bioassay) as that described for oxidized Rous tumor lecithin. Likewise, as with oxidized lecithin, it is seen that citrate salts protected the agent against the inhibitory effects of the oxidized microsome lipides.

Virus inhibition by oxidized methyl linolenate.—The production of a "metabolite" by aerobic incubation of brain, liver, and other tissues, described by Kohn and Liversedge (*5*), was subsequently shown by Bernheim *et al.* (*21*) to be due to the oxidation of the unsaturated fatty acids of those tissues. As was pointed out earlier, these oxidized unsaturated fatty acids are effective inhibitors of cell division, mitochondrial enzyme activity, and respiration and glycolysis in tumor cells. It is apparent also that the oxidized unsaturated fatty acid is the effective portion of the phospholipide (Rous tumor lecithin and microsome lipides) causing virus inhibition.

Although it has not been determined that linolenic acid appears as a constituent unsaturated fatty acid of Rous tumor lecithin or of the microsome lipide fraction, linolenic acid is representative of the nonconjugated polyunsaturated fatty acids of animal tissues. Since the TBA reaction is 30 to 80 times more sensitive for linolenic than for linoleic acid and considerably less sensitive for oleic acid (*4*), linolenate was used to illustrate the effect.

One gm. of the methyl ester was irradiated with ultraviolet light at room temperature for 90 minutes and an aqueous extract of the oxidation products was prepared. After sampling the extract for the TBA reaction, dilutions of it were added to the virus-substrate suspension. One ml. of the diluted extract was added to 4.0 ml. of standard virus preparation CT-632 in 0.05 *M* *p*H 6.0 phosphate or citrate buffer. The mixtures were incubated for 75 minutes at 37° C. Immediately thereafter they were diluted with citrate buffer and assayed for virus activity.

It is obvious from the results shown in part C, text-figure 4, that the presence of oxidized unsaturated fatty acid in the virus suspension greatly inhibited biological activity and that the inhibition was related to the concentration of added oxidation products. It is reasonable to assume, therefore, that the inhibition of virus activity by Rous lecithin and by microsome lipides is due to the oxidation of their constituent unsaturated fatty acids, as measured by the TBA reaction. It is significant that citrates afforded partial protection against the inhibitory effects of the oxidation products up to a certain concentration level (TBA optical density 0.35) of inhibitor. However, when this concentration level was exceeded (*e.g.*, TBA optical density 0.94) virus activity fell off rapidly

to the point where no biological activity could be demonstrated in either the phosphate or the citrate suspensions.

Discussion

The present findings demonstrate that Rous sarcoma virus activity is inhibited by oxidized lipides associated with the virus in the particulate fraction of Rous tumor cells. The inhibition in phosphate buffer is related to the degree of oxidation of the component unsaturated fatty acids (as measured by the thiobarbituric acid reaction), but citrate buffers partially protect the virus against inactivation by relatively high concentrations of the oxidation products. These findings offer a possible explanation of the difference in stability of the virus in the respective buffers, which had been noted in studies previously reported (*12*).

Other investigators have reported similar inhibitory effects on viruses by unsaturated fatty acids. Pirie (*23*) described the effect of pancreas extracts on the Fujinami tumor virus and showed that activity was inhibited by lecithin and fatty acids present in the extract. Begg and Aitken (*24*) pointed out that the use of sodium oleate sometimes leads to regression of the Rous tumor. Likewise, Groupé *et al.* (*25*) showed that sodium oleate had virucidal activity against influenza A and vaccinia viruses. Although none of these investigators refers to degree of oxidation, it is probable that inhibition was related to oxidation of the unsaturated fatty acids employed. It should also be mentioned in this connection that autoxidation of unsaturated fatty acids yields a product which condenses with thiobarbituric acid to form a compound having an absorption spectrum identical to those described herein.

Inhibition of the Rous sarcoma virus by lipide oxidation products (peroxides or aldehydes) is apparently not reversible, since virus activity could not be recovered by removal of the water-soluble oxidation products through stepwise dilution of the inactivated suspensions with citrate buffer. The mechanism of inactivation is still somewhat obscure and further investigations will be required for its elucidation. No -SH groups could be detected by the nitroprusside test, either before or after treatment of the virus suspensions with the lipide oxidation products. The "oxidase" effect of oxidized unsaturated fatty acids on reduced methylene blue, described by Bloor (*20*), may therefore be effected either through the lipide or through the nucleic acid components of the virus.

It is postulated that the mechanism of oxidation of unsaturated fatty acids, as measured by the TBA reaction, involves oxidation at the sites of the double bonds to form a peroxide linkage that is subsequently cleaved to expose an aldehyde group (*4,21*). Thus, Claude's (*2*) concept of spontaneous inactivation of the Rous agent by a chemically active aldehyde group associated with the tumor lipide material is of further significance when one considers the results of the study presented herein and the parallelism between the TBA reaction and the Kreis aldehyde test. Also of significance is the fact that aqueous extracts of the oxidation products of unsaturated fatty acids give a positive test with the Schiff

aldehyde reagent. A final point of interest is Shuster's (*9*) failure to demonstrate the oxidation of lipides in rapidly metabolizing ascites-tumor cells by the TBA reaction. He attributed it to an increase of antioxidant activity in the tumor cells. The suggestion was also made that the presence of oxidation products would inhibit division of the tumor cells [*see also* (*10*)]. These interpretations suggest the possibility of an inverse correlation, at least in the case of the Rous sarcoma, between the degree of malignancy [as measured by rate of tumor growth and recovery of active virus (*18*)] and the presence of oxidized unsaturated fatty acids in the tumor tissue.

The foregoing considerations suggest the possibility that the stability of the Rous sarcoma virus might be increased through the use of appropriate antioxidants. This would be of great advantage in purification studies and in attempts to increase the amounts of active virus recoverable from highly malignant tumors (*26*).

Summary

The oxidation of lipides derived from Rous sarcoma tissue was found to be measurable by the thiobarbituric acid (TBA) reaction of Kohn and Liversedge. With this reaction, a study was made of the effects on standard Rous sarcoma virus preparations of added lipide oxidation products. It was shown that the tumor-producing activity of the virus was inhibited by the oxidation products of Rous tumor lecithin, microsome lipides (phospholipides with the properties of lecithin), and methyl linolenate. The degree of inhibition of phosphate-virus suspensions was related to the concentration of added oxidation products, as measured by the TBA reaction. On the other hand, citrate buffers protected the virus to a large extent against inhibition by relatively high concentrations of the oxidation products of unsaturated fatty acids.

The mechanism of virus inactivation by tumor lipides and application of the findings to the purification problem are discussed.

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The Antibody Response to Successful Tumor Homografts[1]

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That normal somatic tissues can only be successfully grafted into hosts with the same genetic constitution as the graft has been well established. The only nonmalignant tissue reported as showing some degree of homotransplantability is the ovary of the rat (*1*). On the other hand, it has long been known (*2*) that a considerable number of tumors can give successful homografts, *i.e.*, they can grow progressively when grafted into hosts with different genetic constitutions.

In order to analyze whether nuclear changes are involved in this special property of successful homotransplantability, several studies have been undertaken on the relationship between the chromosomes and transplantability of tumors (*3–8*). It has been suggested (*3–5,9*) that chromosomal imbalance in homotransplantable tumors produces a simplified antigenic composition of the tumor cells, which, therefore, produce a decreased antigenic stimulus when grafted into foreign strains. This assumes that due to the decreased antigenic stimulus the host does not produce a strong immune response, thus enabling the tumor to give successful homografts. However, the data have also been interpreted (*6–8*) to show that, although chromosome duplication by itself is not sufficient to give successful homografts, the ability of a tumor to be homotransplantable is accompanied by the presence of duplicate chromosome material in the fundamental tumor cells and (*7,8*) that for some tumors the so-called loss of strain specificity which produces homotransplantability, does not in fact seem to involve the loss of antigenicity.

The development of serologic methods for the detection of hemagglutinins in isoimmune serum of mice (*10–14*) and their application to the analysis of the isoantigenic composition of different mouse strains (*15,16*) have made it possible to determine antibody response to tissue transplants. Based on these methods, some serologic data relating to the suggestion of loss of antigenicity have recently been presented (*9,17*).

The present study was undertaken to determine quantitatively the immune response evoked by tumors with different degrees of homo-

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transplantability. Results from transplantation tests have been correlated with serologic data on antibodies produced by foreign hosts. We have found that homotransplantability, in the tumors studied in the present investigation, is not accompanied by a decrease in the antigenicity of the neoplastic cells. Quantitative analysis of the antibody response has enabled us to show that the homotransplantability of these tumors is due to their resistance to the immune response.

Material

The tumors used in the present study were the ascites forms of the 6C3HED lymphosarcoma and the MC1M and MC1A sarcomas produced by G. Klein (*18*). All 3 tumors originated in C3H mice, and they were maintained by us in the same strain. Unless otherwise stated, the tumors were transplanted after growing in the peritoneal cavity for 7 to 8 days. The C3H, C57BL, and Swiss mice used in our experiments were obtained from the same sources as those used in previous studies (*8*), and the DBA/2 mice were obtained from the inbred colony of the Roscoe B. Jackson Memorial Laboratory.

The 3 tumors used differ in their fundamental chromosome number and homotransplantability (*8*). As shown in table 1, 6C3HED is strain-specific and gives no successful homotransplants; MC1M gives successful intraperitoneal homotransplants but regresses when implanted intramuscularly in foreign strains; MC1A gives successful homotransplants after both intraperitoneal and intramuscular inoculation.

TABLE 1.—*Homotransplantability of 6C3HED, MC1M, and MC1A tumors*

Tumor	Site*	Homotransplantability	
		Strain of origin†	Foreign strain‡
6C3HED	IP	+§	−‖
	IM	+	−
MC1M	IP	+	+
	IM	+	−
MC1A	IP	+	+
	IM	+	+

*IP = Intraperitoneal inoculation; IM = intramuscular inoculation.
†C3H *H-2ᵏ*.
‡C57BR/a *H-2ᵏ*; BALB/c *H-2ᵈ*; DBA/2 *H-2ᵈ*; C57BL *H-2ᵇ*; Swiss *H-2ʳ*.
§+ = Lethal growth of tumor.
‖− = Regression of tumor.

Experimental

State of Immunity Elicited by Successful Homografts

The question of whether MC1M and MC1A show successful homotransplantation at the intraperitoneal site, due to the lack of an immune reaction in the host, was first analyzed by means of simple transplantation tests. The assumption was that if these tumors did not elicit any immune response while growing progressively in hosts of foreign strains, challenging the host with a second graft of the same tumor, after removal of the first graft, should again result in successful tumor growth.

In order to test this, Swiss and C57BL mice were inoculated intraperitoneally with 15×10^6 cells. On the 7th day after inoculation, when the abdomen was quite swollen, the ascites fluid was drawn out with a hypodermic syringe and the peritoneal cavity was rinsed 15 to 20 times with 1 cc. of 0.9 percent NaCl (*19*). Cell counts showed that practically no free tumor cells were left after the 15th rinse. The animals were then reinoculated intraperitoneally with the same tumor. Similar experiments were carried out in which the intraperitoneal inoculation was followed by an intramuscular one. There is, of course, the possibility that with this method of tumor removal, cells attached to the peritoneum or infiltrating the subperitoneal tissue may remain in the host. However, when the same method was used in the strain of origin (C3H) there was no regrowth of tumor.

The results, summarized in tables 2 and 3, show that in the strain of origin a second intraperitoneal graft of the same tumor always resulted in progressive growth of the second inoculum and death of the host. In foreign strains, such a second inoculation resulted in complete regression of the graft. Similar results were obtained with intramuscular

TABLE 2.—*Behavior of second MC1M and MC1A transplants after removal of a first, intraperitoneal inoculation of same tumor*

Tumor	Site of second inoculation	No. mice with tumors / Total no. mice		
		C3H	C57BL	Swiss
MC1M	Control*	10/10	10/10	10/10
	IP	10/10	0/10	0/10
MC1A	Control*	10/10	10/10	10/10
	IP	10/10	0/10	0/10
MC1A	Control†	10/10	10/10	10/10
	IM	10/10	0/10	0/10

*Received only 1, intraperitoneal, inoculation.
†Received only 1, intramuscular, inoculation.

transplantation preceded by intraperitoneal inoculation—MC1A, which otherwise grows intramuscularly in foreign strains, was no longer able to grow progressively in this site. When MC1M was inoculated intramuscularly into hosts of foreign strains, it grew for about 8 days and then regressed, but when grafted into this site after intraperitoneal inoculation, no signs of growth could be detected (table 3).

TABLE 3.—*Behavior, in Swiss mice, of intramuscular MC1M transplants inoculated following removal of first, intraperitoneal transplants*

Group	Day after second inoculation:*			
	3	6	10	20
Controls†	0/10	10/10	3/10	0/10
Mice with second transplants	0/10	0/10	0/10	0/10

*The numerator shows the number of tumor-bearing mice. The tumors later regressed.
†Received only 1, intramuscular, inoculation.

One can conclude from these experiments that the first graft in a foreign strain produces a definite state of immunity in the host, which is functionally manifested only by a subsequent tumor graft. Had no immune response been induced by the first transplant, the second transplant should have grown progressively.

On the basis of this conclusion 2 types of immune systems can be postulated to explain the regression of the second graft. One is based on the assumption that the successful intraperitoneal growth of a single tumor graft, either MC1A or MC1M, is due to the weakness of antigenicity of these tumors. Accordingly, one would postulate that the first transplant elicits only a very low immune response, much weaker than that necessary for tumor destruction, and it is only the second inoculation that raises the immune reaction to a secondary response. It has been shown (*20*) that 1 intravenous injection of staphylococcal toxoid produced ". . . practically no formation of anti-toxin, although it rendered the animal capable of giving a typical sharp secondary response to the next injection . . . ." (*21*).

An alternative explanation does not necessarily relate the growth of tumors in foreign strains to the low antigenicity of the tumor cells. It can be supposed that the first inoculum elicits a rather high immune response due to a strong antigenic stimulus of the tumor, and that the tumors have some special mechanism of resistance toward the antibody response connected with the growth properties and the pattern of antigen synthesis peculiar to them. The first graft can then overcome the immune response produced by itself, while a second graft, because of its small number of cells, is devoid, during this period, of the capacity to resist the preformed antibody response, and it therefore regresses. These possibilities are just 2 extremes of a wide range of possible variations.

In order to differentiate between these possibilities, a quantitative analysis of the immune reaction of the 3 tumors, 6C3HED, MC1M, and MC1A, was carried out.

Hemagglutinins Produced by Tumors with Different Degrees of Homotransplantability

Methods

The serologic technique for hemagglutinin determination of Gorer and Mikulska was used (*13*). In all 3 tumors antisera produced against tumor cells were tested on erythrocytes from C3H mice. The dilutions of antisera were carried out in 3 percent dextran (Intradex, salt-free, Glaxo) and the erythrocytes were suspended in normal human serum, diluted 1:0.5 in saline. The human serum was heat-inactivated and then stored at −20° C. Before use each sample was absorbed with mouse erythrocytes and then suspended with C3H red blood cells to a concentration of 2 percent. The red-cell suspension and antisera were incubated in precipitin tubes at 37° C. for 1 hour and then read.

In our experiments the importance of normal human serum in activating agglutination of red blood cells was demonstrated by testing antisera produced in Swiss mice after 3 inoculations of 6C3HED. The dextran-diluted antiserum was tested for agglutinins in a saline suspension of C3H erythrocytes and compared with erythrocytes suspended in normal human serum. It was found that, whereas in the saline suspension the titer of antibody dilution was 1:64, the erythrocytes in human serum gave a titer of 1:2048. We also found that the same inoculum of tumor cells produced different intensities of antibody response in different strains. Thus Swiss mice formed a titer of $1:2^{11}$ against 3 inoculations of 6C3HED, while in the DBA/2 mice the titer was $1:2^{7}$. It should also be mentioned that due to differences in origin and the length of storage at −20° C., different samples of human serum produced varied titers, so that for each experiment we used the same sample of human serum for all tests.

Since, in ordinary inoculations of ascites tumors, some erythrocytes are transferred with the tumor cells, and since the amount may vary from one inoculum to another, tests were made to check whether the agglutinogens of these erythrocytes contributed to the antigenic stimulus of the tumor graft. Two groups of 10 Swiss mice each were injected, one with 0.1 cc. of a 10 percent C3H erythrocyte suspension in saline, the other with 0.2 cc. of the same suspension. These concentrations of blood cells are well above the maximal concentration that may be found in the ascites fluid inoculated in our experiments. Sera collected from these mice did not reveal any agglutinins when tested by the dextran-human serum method.

Hemagglutinins Produced after 1 Inoculation of 6C3HED, MC1M, and MC1A

We first attempted to compare the immune reactions elicited by 1 inoculation of tumor cells.

Three groups of Swiss mice (8 animals per group) were injected intraperitoneally with 6C3HED, MC1M, and MC1A, respectively. The ani-

mals were bled 11 days after inoculation, and the pooled antisera were tested for hemagglutinins. The results (table 4) show that MC1A produced a much stronger antibody response than the other 2 tumors. MC1A was the most homotransplantable tumor among the 3 and produced the highest immune response but this correlation between transplantability and agglutinin production was not found for the other 2 tumors in this experiment.

Since Swiss mice show some individual variation in their reaction against tumor grafts, further experiments were carried out with C57BL as hosts.

The 3 tumors were injected intraperitoneally into C57BL hosts and hemagglutinins were determined at various time intervals after inoculation. In order to eliminate the effect of bleeding on subsequent antibody concentration, different mice were bled each time, so that each animal was bled only once during the experiment. Since the previous experiment had shown that the homotransplantable tumors produced hemagglutinins while growing progressively, the possibility of *in vivo* absorption of antibody by the tumor antigens was anticipated. Furthermore, it was suspected that the comparatively low titer produced in Swiss mice by MC1M was due to the elimination of free antibodies by the growing ascites tumor. It seemed, therefore, that in order to estimate the total production of antibodies, or at least the efficiency of antibody production stimulated by a tumor, one ought to add to the free agglutinins of tumor-bearing mice the amount absorbed by the tumor antigens. As a partial solution to this difficulty, the tumors were removed 7 days after inoculation, and agglutinins were determined at various times afterward. It is obvious that, although the concentration of free agglutinins in animals without tumors is higher than that in corresponding tumor-bearing mice, it is still lower, at least up to a certain period, than the *total* amount of *free* and *bound* antibodies produced at a given time. It will be shown later that the presence of antigen and its continued synthesis increases, up to a certain time limit, the production of antibodies in tumor-bearing mice, while the removal of the antigenic material probably results in a somewhat lowered antibody production. Nevertheless, the values obtained after removal of the tumors seem to reflect the immune response more efficiently than other methods of estimation.

The hemagglutinins produced after 1 intraperitoneal inoculation of each of the 3 tumors are given in text-figure 1. Each point on the curve represents the results with pooled sera from 5 mice. Since 6C3HED regresses about 8 days after inoculation, the values for this tumor are comparable to those for MC1M and MC1A that had been removed.

The main points shown by the hemagglutinin curves of the intraperitoneal tumors are the following:

1) The rates of antibody production in the mice in which the tumors were no longer present can be expressed in the following order: MC1A> MC1M>6C3HED. While the maximal titer of 6C3HED was 2^6, that of MC1M was 2^8, and MC1A reached a titer of 2^{11}. Furthermore, antibody production following removal of the tumor lasts for a longer period

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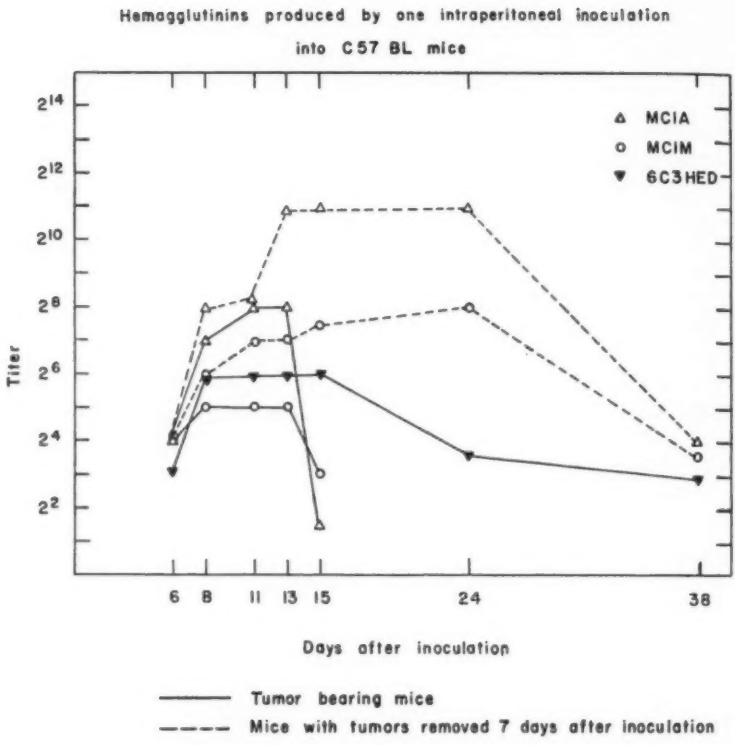
TABLE 4.—*Hemagglutinins produced by 1 intraperitoneal inoculation of 6C3HED, MC1M, and MC1A into Swiss mice*

Tumor	Antiserum dilution 1:									
	2	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}
6C3HED	++	++	++	++	+	−	−	−	−	−
MC1M	−	−	+	±	−	−	−	−	−	−
MC1A	++	+++	+++	+++	+++	+++	+++	++	++	+
Control: Normal Swiss serum	−	−	−	−	−	−	−	−	−	−



TEXT-FIGURE 1.—Hemagglutinins produced by 1 *intraperitoneal inoculation* of tumors with different degrees of homotransplantability.

in MC1A and MC1M than that in 6C3HED. In the latter, the decline starts between the 15th and the 24th day, while in the other 2 tumors it falls only after the 24th day. It appears, therefore, that for these 3 C3H tumors the *higher the degree of homotransplantability, the stronger the isoagglutinin reaction.*

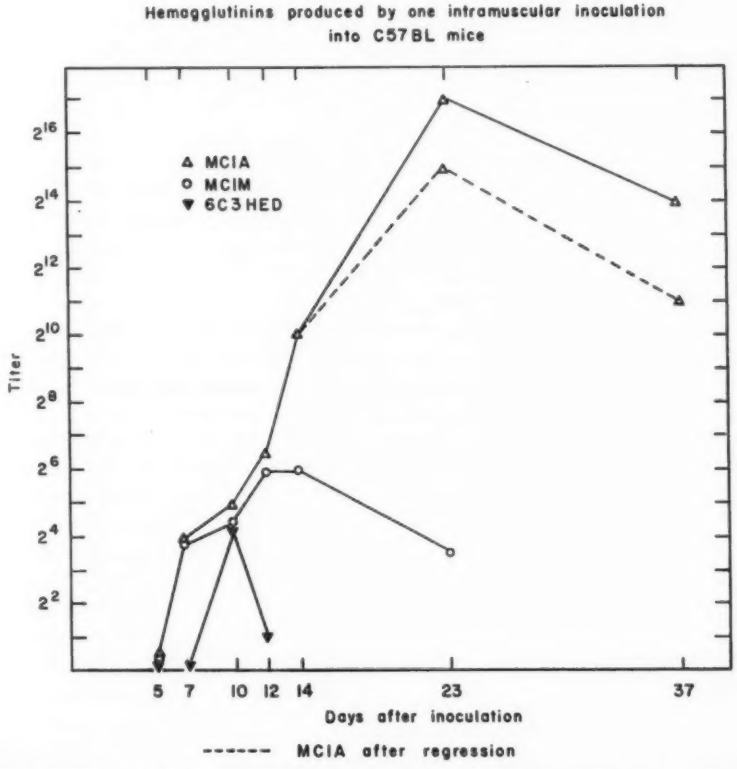
2) Removal of the growing tumors is followed by an immediate increase in free agglutinins. It is therefore obvious that while the tumor is growing, its isoantigens remove free antibodies from the circulation.

3) The decline of the descending arms of the curves for tumor-bearing mice is inverse to the rise of the ascending arms of the curves for the mice that no longer had tumors. Whereas in the latter animals MC1A reaches a higher peak on the 15th day than MC1M, in the mice with tumors the decline is steeper in MC1A than in MC1M and MC1A reaches a lower titer on the 15th day. Since during the period of the apparent decline of free antibodies in tumor-bearing animals the mice without tumors show an increase, it follows that the *rate of neutralization of free isoantibodies by their homologous isoantigens is stronger in MC1A than in MC1M.* Since this neutralization is carried out by the isoantigens of the tumors, the rate

of the neutralization process, which is an *in vivo* antigen-antibody reaction, is determined by the amount of antigen available for antibody absorption, *i.e.*, the rate of antigen production by the tumor. It can therefore be concluded that the rate of antigen production is higher in MC1A than in MC1M and that it is higher in MC1M than in 6C3HED.

4) The intensity of the immune reaction is determined by the amount of isoantigen produced by the tumor cells. This follows directly from the results with the tumors growing intraperitoneally, from which it was concluded that MC1A produced more antigen than MC1M and 6C3HED; those results also indicated that, of the 3 tumors tested, MC1A elicited the highest agglutinin response.

Hemagglutinin curves were also obtained after a single intramuscular inoculation of each of the 3 tumors (text-fig. 2). Each point on the curve represents the results from 5 mice. At this site 6C3HED and MC1M regressed in foreign strains, whereas MC1A normally grew progressively. It was found that under these conditions the values for MC1A reached a much higher level than those for the other 2 tumors. This can be ex-



TEXT-FIGURE 2.—The hemagglutinins produced by 1 *intramuscular inoculation* of tumors with different degrees of homotransplantability.

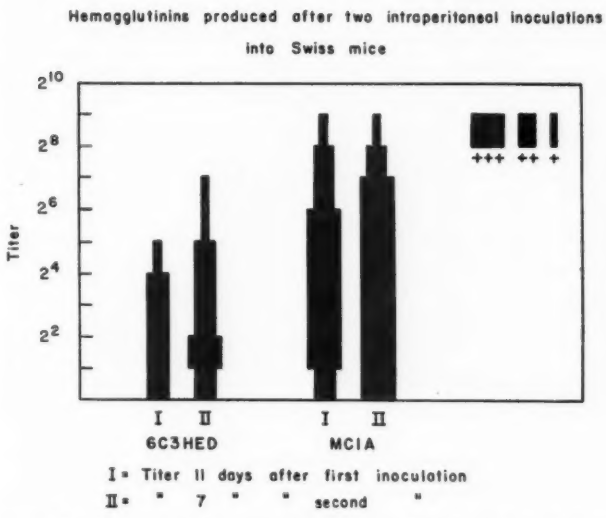
plained by the persistence of the MC1A tumor, *i.e.*, the continuous production of antigens, and in fact the rise in the agglutinin curve of MC1A, after the 12th day, occurred when MC1M and 6C3HED had already completely disappeared. Similar results for another tumor were previously reported by Gorer (*11*). It is of interest that in 5 MC1A mice, which had been bled on the 14th day, the tumor regressed after the bleeding. The agglutinin curve for these mice is also given in text-figure 2 in the form of a dotted line. It can be seen that the titers of these animals, although still very high, were lower than those of mice in which the tumor continued to grow.

Hemagglutinins Following 2 Inoculations of 6C3HED, MC1M, and MC1A

The state of transplantation immunity evoked by 1 tissue graft is usually evaluated by testing the reaction of the host toward a second graft. The decrease in the survival time of the second graft indicates that an immune response was elicited by the first transplant (*22–24*). It is customary to consider the decrease in the survival time of the second graft as a secondary response to the host (second-set effect), *i.e.*, an increase of the immune response of the host fundamentally similar to that obtained by a second injection of antigen in classical immunology (*21*). The results reported for our first transplantation tests (table 2) may seem at first inspection to belong to this category, since a second graft of MC1A does not show merely a decrease in survival time; rather, the tumor changes from one with an "infinite survival time" at the first graft, to one which does not survive more than 10 days. A quantitative determination of isoantibodies shows, however, that regression of the second graft is not accompanied by a real secondary response for isohemagglutinins.

A preliminary experiment was carried out in Swiss mice. Two groups, of 10 animals each, were given 2 inoculations of tumor cells at a time interval of 9 days. One group was inoculated with 6C3HED, the other with MC1A. Eleven days after the first inoculation, and 7 days after the second, the animals were bled and the hemagglutinins determined. The results, summarized in text-figure 3, show that a second graft of 6C3HED increases, to some extent, the antibody titer of the first. No real difference was found between the agglutinin concentrations following 1 or 2 inoculations of MC1A. With MC1A, the tumors were removed 7 days after the first inoculation.

This experiment was then extended with C57BL as hosts. The animals were divided into 2 series, one (A), with a 7-day interval, the other (B), with a 14-day interval between the 2 inoculations. Series A and B consisted of 3 groups each, according to the first tumor graft, which was either 6C3HED, MC1M, or MC1A. Each of the 3 groups was further divided into subgroups of 8 animals each, according to the second tumor graft; a control group, which received only 1 inoculation (comparable to the first graft of the experimental animals), was added. In all the mice, including those inoculated with 6C3HED, the tumors were removed on the 7th day after the first inoculation and the mice were bled 10 days



TEXT-FIGURE 3.—Hemagglutinins produced after 2 *intraperitoneal inoculations* of 6C3HED and MC1A. The second inoculation was given 9 days after the first.

after the second inoculation. The controls were bled with the animals of their corresponding subgroups, *i.e.*, 17 days after inoculation in series A, and 24 days in series B. The results are given in table 5.

The MC1A group.—Hosts inoculated first with MC1A and then challenged with a second tumor graft did not show any increase in titer as compared with animals given just 1 inoculation of MC1A. In addition, the same isoantibody concentration was found in both the 7- and 14-day series. It seems, therefore, that an additional tumor graft preceded by MC1A, within the given time intervals and of the given inoculum size (15×10^6 cells), did not increase the immune response of the hemagglutinins. If we postulate that the rate of hemagglutinin production is similar to that of the cytotoxic isoantibodies, we may draw the following conclusion about the behavior of the tumor grafts. Since an intraperitoneal inoculation of either MC1M or MC1A preceded by MC1A did not grow progressively, and since during their regression no increase in antibody response was detected, it was the immune reaction evoked by the first graft that prevented progressive growth of the second. Accordingly, the regression of the second inoculation, which was not accompanied by a real increase in host resistance to the graft, was not accompanied by a true secondary response. It can, therefore, be concluded that the fact that the first MC1A graft does grow in a foreign strain cannot be accounted for by a low antigenic stimulus of the first graft. On the contrary, the first graft gives a strong stimulus followed by a strong response.

The MC1M group.—In the 7-day series, the MC1M and MC1A inoculated animals did not seem to differ much in their agglutinin titers, nor

TABLE 5.—*Hemagglutinin titers following 2 tumor inoculations into C57BL mice*

Series A Second inoculation 7 days after first			Series B Second inoculation 14 days after first		
First graft	Second graft	Titer	First graft	Second graft	Titer
MC1A	—	2^{14}	MC1A	—	2^{14}
MC1A	MC1M	2^{14}	MC1A	MC1M	2^{14}
MC1A	MC1A	2^{14}	MC1A	MC1A	2^{14}
MC1M	—	2^{8-10}	MC1M	—	$2^{6?}$*
MC1M	MC1M	2^{9}	MC1M	MC1M	2^{12}
MC1M	MC1A	2^{10}	MC1M	MC1A	2^{8}
6C3HED	—	2^{8}	6C3HED	—	2^{4-6}
6C3HED	6C3HED	Not tested	6C3HED	6C3HED	2^{11}
6C3HED	MC1M	2^{9}	6C3HED	MC1M	2^{13}
6C3HED	MC1A	2^{11-12}	6C3HED	MC1A	2^{14}

*End point not clear.

did they show a stronger immune reaction than animals with only 1 inoculation of MC1M. This situation is similar to that in the MC1A group.

In the 14-day series, the animals reinoculated with MC1M had higher titers than the corresponding subgroup of the 7-day series and, of course, higher than the MC1M controls tested after 24 days. In other words, there is some secondary response in this group.

In the MC1A subgroup of the 14-day series, the picture is not entirely clear. In this group the second graft of MC1A grew, and the ascites fluid was rinsed out 1 day before bleeding. The titer of 2^8 is probably a residual free antibody left after a rather high rate of agglutinin absorption by the tumor cells. It is, of course, impossible to estimate the total amount of antibody production based on the residue of 2^8, but in comparison with previous data (text-fig. 1), it seems quite safe to estimate that the total titer could be at least as high as, and probably higher than, that of the MC1A subgroup of the 7-day series.

The 6C3HED group.—In this group the difference between the 2 series, A and B, is clearer than in the MC1M group. In series A there was some increase in antibody response in the experimental subgroups as compared with the controls. Furthermore, a second inoculation of MC1A raised the titer more than a second inoculation of MC1M.

In conclusion, assuming again that the agglutinin concentrations reflect the intensity of the host's resistance to tumor grafts (evidence for this assumption will be given in a later section), the following may be noted. Whereas a first inoculation of MC1A had the strongest effect in inhibiting growth of second tumor grafts, of both MC1M and MC1A,

neither of the second grafts took an active part in eliciting the immune reaction to which they succumbed. A real secondary response was observed primarily with 6C3HED and to a lesser extent with MC1M. Thus the tumor that was the weakest in its primary antigenic stimulus gave the most pronounced secondary response. It also appears that within certain limits, the greater the time interval between the 2 inoculations, the higher the secondary response.

Effect of Dosage and Site of Inoculation on Isoantibody Production

The above experiments have made it clear that the time interval between 2 tumor grafts may be important in the final production of isohemagglutinins. Other experiments were carried out in which the time interval between the 2 inoculations was decreased to zero, so that the only variable factor was the inoculum size. This was done by challenging animals either with 2 simultaneous inoculations in different sites or with inoculations of different doses of tumor cells at the same site.

One group of 10 C57BL mice was inoculated with 15×10^6 cells of MC1M in 1 hind leg, and a second group of 10 was inoculated with the same number of cells in each hind leg. Hemagglutinins were tested after 13 days, and the first group had a titer of 2^4, while the second reached a titer of 2^7 (table 6). That this result was not due to the larger total inoculum in the animals injected in both legs was shown by inoculating another experimental group with 30×10^6 in 1 leg. The agglutinin levels of the latter group (table 6) were identical to those inoculated with 15×10^6 cells.

It seems most probable that the increase in the immune response due to inoculation in 2 legs is a function of an additive response of local lymph

TABLE 6.—*Effects of dosage and site of inoculation on production of hemagglutinins by MC1M*

Site of inoculation	Size of inoculum	Titer
IP	45×10^6	2^9
IP	30×10^6	2^{11}
IP	15×10^6	2^{13}
IP IM	15×10^6 15×10^6	2^{13}
IM 1 leg	15×10^6	2^4
IM 2 legs	15×10^6	2^7
IM 1 leg	30×10^6	2^4

nodes. If the lymph nodes close to the grafted leg are mainly involved in the antibody production (*24,25*), a graft into the second leg will induce other lymph nodes to produce antibodies, which are emitted into the circulation and detected as a higher agglutinin concentration.

However, with intraperitoneal inoculation, where the tumors grow progressively, the situation seems to be different. Two groups, of 10 C57BL each, were inoculated with MC1M. In the first group each animal received 2 simultaneous injections (15×10^6 cells each), one intraperitoneally and the other intramuscularly. In the second group, mice were given only 1 intraperitoneal inoculation of 15×10^6 cells. The intraperitoneal tumor was removed on the 7th day and hemagglutinins were determined 1 week later. Although it might be that, in this experiment too, each of the simultaneous tumor grafts acts on different lymph nodes, no difference in the antibody titer was found between the 2 groups (table 6). It may be suggested tentatively that the tumor growing progressively in the intraperitoneal site swamps the host with antigenic material, causing a functional saturation of the antibody-forming system and therefore an additional intramuscular graft did not raise the amount of agglutinins.

The effect of dose in intraperitoneal inoculations was also tested. Three groups, of 8 C57BL mice each, were inoculated with 15×10^6, 30×10^6, and 45×10^6 cells, respectively. The tumors were removed on the 7th day and the animals were bled on the 13th. As shown in table 6, not only did a higher dose not produce a higher agglutinin level at the time it was tested, but, in fact, with larger inoculums lower titers were found. Two assumptions can be made to explain this apparent inverse relationship between inoculum size and agglutinin titer. 1) A higher dose of ascites-tumor cells, in causing a shorter lag period in tumor growth (*19*) may induce an earlier rise of the ascending arm of the agglutinin curve, which may be followed by an earlier decline of the descending arm. It may be due to this last phase that fewer agglutinins were found with larger inoculums of tumor cells. 2) A higher dose of cells may elicit more antigens from the tumor, and when these antigens are drained into the reactive lymph nodes, they may, after reaching a certain concentration, absorb the newly formed antibodies and thus prevent their release into the circulation. If a higher cell dose induces a higher rate of antigen excess in the lymph nodes, it will cause a higher rate of antibody fixation in the nodes and a lower concentration of free agglutinins. (Both explanations are now under investigation.)

Isohemagglutinin Response as a Reflection of Intensity of Host Resistance to Tumor Grafts

It seems quite certain that the isohemagglutinins produced against a tumor graft are not the isoantibodies responsible for the destruction of tumor transplants (*10,26,27*). The latter type of antibodies are, of course, those which represent the real resistance of the host to tissue transplants, and although it has been shown for leukemia (*28*) that the cytotoxic antibodies may, like the free hemagglutinins, exist as free antibodies in

the host's serum and can be passively transferred and immunize other mice, these antibodies seem to be distinct from hemagglutinins. Mice normally resistant to a certain tumor can be made susceptible to this tumor by pretreatment with lyophilized tissues. In this case no cytotoxic antibodies were demonstrated in the host's serum, although hemagglutinins have clearly been shown (*26*) to be present. Before any conclusions are drawn about the validity of the evidence obtained from agglutinin determinations to explain phenomena of tumor transplantability, one ought to test whether under the particular experimental conditions being investigated host resistance to tumor grafts does coincide with the isohemagglutinin reaction. We have made some transplantation tests that indicate that the intensity of the agglutinin reaction is similar, in untreated mice, to the intensity of the hosts' resistance to the 3 tumors studied.

Cytotoxic immunity produced by MC1A and MC1M.—MC1A was found to elicit stronger hemagglutinin immunity than MC1M. The question of whether this tumor also produced a higher degree of cytotoxic immunity was answered in the following experiment. Two groups of Swiss mice and 2 groups of C57BL mice were inoculated intraperitoneally with MC1A; in one group this inoculation was preceded by inoculation of MC1A and in the other by MC1M. The first grafts were removed on the 7th day. Two other groups were similarly inoculated with MC1M, preceded by MC1M and MC1A. The results (table 7) show that preimmunization of foreign strains with MC1M provokes host resistance that inhibits the growth of a subsequent graft of MC1M, but it is still too weak to destroy a subsequent graft of MC1A. However, preimmunization by MC1A will prevent the growth of both MC1M and MC1A. It follows, therefore, that *MC1A produces a higher cytotoxic immune response than MC1M, just as it produces a higher hemagglutinin response than MC1M.*

Intramuscular inoculation of MC1M produced a *lower* and *shorter* agglutinin response than an intraperitoneal inoculation of MC1M, and it can be shown that there is a similar relationship for the "cytotoxic

TABLE 7.—*Immunizing effect of intraperitoneal inoculations of MC1M and MC1A on second intraperitoneal inoculations of these tumors*

Transplants	C3H*†	Swiss*	C57BL†
1. MC1A 2. MC1A	10/10	0/10	0/8
1. MC1M 2. MC1A	10/10	10/10	8/8
1. MC1M 2. MC1M	10/10	0/10	0/8
1. MC1A 2. MC1M	10/10	0/10	0/8

*Seven-day interval between first and second graft.
†Fourteen-day interval between first and second graft.

antibody" response of this tumor. Two groups of C57BL mice were inoculated with MC1M, one with an intramuscular, the other with an intraperitoneal inoculation of the same size (15×10^6 cells). Seven days after the first graft the intraperitoneal tumor was rinsed out and both groups were given a second intraperitoneal inoculation of the same tumor. The results (table 8) show that while an intraperitoneal tumor immunized the animal to the extent that a second intraperitoneal graft would not grow progressively, an intramuscular tumor produced a much weaker immune response, which enabled a second intraperitoneal graft to grow progressively and kill the host. These results also coincide with the data obtained by agglutinin determinations.

TABLE 8.—*Immunizing effect of intraperitoneal and intramuscular inoculations of MC1M on a second intraperitoneal inoculation of MC1M*

Transplants*	Route	C3H	C57BL
1. MC1M 2. MC1M	IP IP	8/8	0/8
1. MC1M 2. MCIM	IM IP	8/8	8/8

*Seven-day interval between first and second graft.

Secondary response.—It has been shown that MC1M and MC1A did not stimulate any secondary response of isohemagglutinins when the second graft was inoculated 7 days after the first. It is only when the time interval between grafts increases that the second application of antigenic material may succeed in eliciting heightened "agglutinin resistance" in the host. Similar results were found for antibodies involved in tumor destruction. The experiment just described shows that an intraperitoneal inoculation of MC1M, given 7 days after an intramuscular graft, resulted in successful homografts. However, when the time interval between these 2 inoculations was increased to 14 and 21 days only 1 out of 6 grew progressively, while the rest developed increased immunity which caused a complete regression of the intraperitoneal tumor graft (table 9). Other results in support of this conclusion have been reported previously (*8*).

In view of these transplantation tests, it may be stated that in untreated normal hosts the immune response of isohemagglutinins, elicited by the tumors investigated, parallels the state of tumor-transplantation immunity.

In vitro *absorption of isohemagglutinins by 6C3HED, MC1M, and MC1A tumor cells.*—Experiments described in prior sections have shown that each of the 2 homotransplantable tumors grown in a site that normally gives lethal homotransplants can immunize against any of the other tumors. Similar results have been obtained in tests on the immunizing effects of intramuscular and subcutaneous inoculations of these tumors (*8*). These experiments clearly show that the tumors share some isoantigens that function in transplantation immunity. It has been postulated

TABLE 9.—*Effect of time interval between 2 MC1M inoculations on secondary response produced by second inoculation*

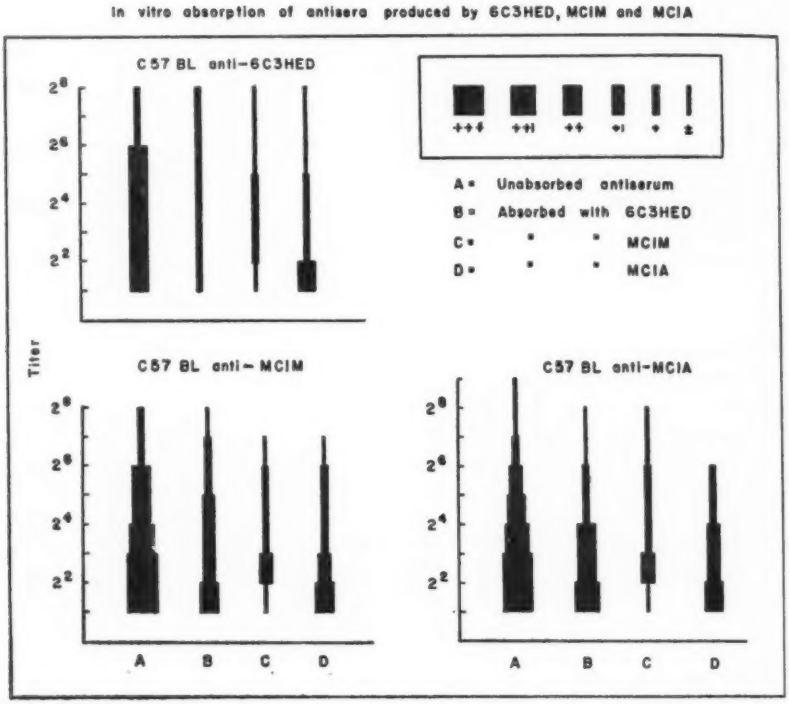
Interval between 1st (IM) and 2nd (IP) inoculation (*days*)	C3H	C57BL
7	8/8	8/8
14	6/6	1/6
21	6/6	1/6

(*9*) that tumors capable of successful homotransplantation are those that have lost their strain specificity through loss of antigenicity. Since the attempt to demonstrate this loss of antigenicity was performed by *in vitro* absorption experiments for hemagglutinins (*9,17*), we have carried out such tests in order to demonstrate the *in vitro* capacity of our homotransplantable tumors to absorb antibodies produced by the strain-specific 6C3HED, and *vice versa*.

Isoimmune sera against 6C3HED, MC1M, and MC1A were produced in C57BL mice and tumor cells for absorption were taken from 7-day-old ascites fluid of the 3 tumors grown in C3H mice. The fluid was mixed with 4 percent sodium citrate in saline, then washed and centrifuged 5 times in order to remove the erythrocytes from the tumor cells. The tumor cells were finally suspended in saline at concentrations of 60×10^6, 45×10^6, and 120×10^6 cells per cc. for MC1A, MC1M, and 6C3-HED, respectively. Each type of cell suspension was mixed with an equal volume of antiserum, and unabsorbed sera were mixed with equal volumes of saline as controls. One absorption was carried out at 6° C. for 3 hours with each batch of serum, after which the cell-serum suspensions were centrifuged and tested for agglutinins against C3H red blood cells.

The results (text-fig. 4) show that although absorption was not complete, MC1M and MC1A cells fixed agglutinins from each of the 3 isoimmune sera. In fact, from antisera produced against MC1M and MC1A, cells of the homotransplantable tumors show a stronger tendency to absorb hemagglutinins than cells of 6C3HED.

In another experiment with antiserum against 6C3HED, produced by 2 intraperitoneal inoculations, the incubation time of tumor cells with antibody was increased and each sample of antiserum was absorbed twice. The first absorption was performed according to the method described above, but the cells were left with the antiserum overnight at 6° C. The antisera-tumor cell suspensions were then centrifuged and new samples of tumor cells, suspended in 2 percent dextran, were added and incubated for 2 hours at room temperature. Agglutinin determinations carried out after the second absorption revealed complete removal of hemagglutinins by cells of each of the 3 tumors (table 10).


In vitro absorption of antisera produced by 6C3HED, MCIM and MCIA
C57 BL anti-6C3HED
C57 BL anti-MCIM
C57 BL anti-MCIA
Titer
A = Unabsorbed antiserum
B = Absorbed with 6C3HED
C = " " MCIM
D = " " MCIA
A B C D

TEXT-FIGURE 4.—Results of a single *in vitro* absorption with cells of the 3 tumors.

TABLE 10.—*Complete* in vitro *absorption of hemagglutinins from C57BL anti-6C3HED serum by 6C3HED, MC1M and MC1A cells*

Serum	Titer
Unabsorbed	256
Absorbed with:	
6C3HED	0
MC1M	0
MC1A	0

These results clearly demonstrate that all 3 tumors are capable of *in vitro* absorption of hemaggluttinins from antisera produced by their own and the other 2 tumors.

Discussion

Antigenic Composition and Antigenic Stimulus

The interest in the capacity of tumor cells to give successful homografts derives not only from the fact that the analysis of this phenomenon may reveal some new principles concerning the immunogenetic behavior of

cells, but also, since it is a phenomenon confined to neoplastic tissues, that it may provide information on some special properties that are specific to tumors. It is perhaps the very fact that homotransplantability is found only among neoplastic tissues that has directed previous interpretations toward the suggestion of antigenic simplification, *i.e.*, loss of antigens, etc. Neoplastic tissues are known to show various degrees of morphologic dedifferentiation; as for organ specificity, evidence has also been presented to show that there may be some biochemical dedifferentiation that is expressed by the loss of organ antigens (*29*). The hypothesis of antigenic simplification as the basis for homotransplantability would, therefore, seem to be in harmony with the aforementioned properties of tumors. The idea that during transplantation there may be an increase of some antigens and that one or more antigens may crowd out the others (*30*) suggests one of the possible changes that may have taken place in homotransplantable tumors.

Before drawing conclusions as to the antigenic composition of tumors, one should be aware that it is not the antigenic complexity (as opposed to simplicity) that determines the hosts' resistance to tissue transplants but rather the intensity of the antigenic stimulus (= antigenicity) that determines the strength of the immune response. A simplification of the antigenic complex, *i.e.*, loss of some antigenic components, does not necessarily result in a decrease of antigenicity if the latter is measured by the intensity of the antibody response. On the contrary, it has been shown from many systems, such as diphtheria toxins (*31*) and pneumococcus types (*32*), that the more complex the antigenic material injected, the less intense is the antibody response. The highest immune reactions in these cases were obtained with 1 type of toxin or with not more than 3 types of pneumococcus. A loss of antigens, therefore, does not necessarily indicate a decrease in antigenicity. For this reason, in our study we have given special attention to the antigenicity of homotransplantable tumors.

Antigenicity and Transplantability of Tumors

In order to analyze the antigenic stimulus produced by tumor grafts, we have used 2 complementary approaches: transplantation tests and hemagglutinin determinations. Both have shown that the antibody response produced by 1 tumor inoculation was of the following order of intensity: MC1A>MC1M>6C3HED. The strain-specific tumor 6C3HED elicited a much lower and less persistent hemagglutinin titer than the other homotransplantable tumors. That there is an *in vivo* absorption of antibodies has been clearly demonstrated by the sharp increase in agglutinin titer after removal of a growing tumor. It has also been shown that the extent of *in vivo* absorption differs in the 3 tumors.

After removal of the tumors, isohemagglutinins in the anti-MC1A serum reached a higher level than in the anti-MC1M serum in the same way that MC1A produced a higher level of host resistance. However, when these 2 tumors were allowed to grow progressively in the foreign

host, the values for MC1A agglutinins were lower than those of MC1M. In presenting the results schematically, the agglutinin titers of the tumor-bearing mice are designated by the symbol *tb*, and those of the mice whose tumors have been removed by the symbol *tr*. The designations for the 2 homotransplantable tumors, then, are MC1A *tb*, MC1A *tr*, MC1M *tb*, and MC1M *tr*. Since it has been shown that:

$$\text{MC1A } tr > \text{MC1M } tr \quad \text{(text-fig. 1)}$$

but that

$$\text{MC1M } tb > \text{MC1A } tb \quad \text{(text-fig. 1)}$$

it follows that

$$\text{MC1A } tr - \text{MC1A } tb > \text{MC1M } tr - \text{MC1M } tb.$$

Since $tr-tb$ represents the antibodies absorbed by the antigenic material of the tumor, it follows that the rate of absorption by MC1A is higher than by MC1M. However, these values also represent the amount of antigen available for absorption by the tumor. It can therefore be inferred that the amount of functional antigen produced during progressive growth is larger in MC1A than in MC1M.

The production of a larger amount of antigenic material by MC1A may explain 2 phenomena which at first seem contradictory: 1) the greater immune response evoked by MC1A, *i.e.*, the greater antigenicity of this tumor; 2) homotransplantability, which in our experiments is progressive growth in an environment that is, immunologically, strongly reactive. The higher production of antigen can explain homotransplantability if one assumes that when a higher amount of antigenic material is involved in eliciting antibody response by tissue grafts, extracellular antigen is the active component. If this is the antigenic material that also absorbs antibodies, which is a reasonable assumption, then the greater the emission of antigen, the higher the rate of isoantibody fixation. It follows, therefore, that by this mechanism, antibodies are neutralized outside the cells, leaving the tumor cells untouched. The extracellular emission of isoantigens has also been postulated (a) in order to explain the results with grafts of normal tissues (*27*) and (b) as possibly playing a role in tumor homotransplantability (*33*).

We have obtained additional evidence (unpublished) on the role of extracellular proteins in tumor homografts from experiments in which radioactive amino acids were used for the study of protein emission from cells. The greater rate of antigen production may also be related to the role of gene duplication in homotransplantability (*3–5*).

Homotransplantability and Resistance to Antibody Response

The vital function of intercellular antigen in antibody absorption and hence in homotransplantability may be deduced indirectly from a comparison of antibody production by solid and ascites forms of MC1A. At the intraperitoneal site the tumor grows in an ascites form, whereas intramuscularly it grows as a solid tissue. In the ascites form, the tumor

cells are suspended in a substance that is at least partly formed by the cells, while in the solid form, in which there is a minimal amount of intercellular space, substances emitted by the cells are presumably secreted largely outside the whole tissue and removed by the circulation. It is probably due to these differences that the absorptive capacity of the solid form is much weaker than that of the ascites form. Removal through regression of an intramuscular tumor, unlike removal of the intraperitoneal ascites tumor, did not increase the agglutinin titer. The fact that solid forms, such as the intramuscular MC1M transplant, are less resistant than an intraperitoneal MC1M transplant may, accordingly, be attributed to the lack of a sufficient amount of intercellular antigen. This may also apply to differences between intramuscular and subcutaneous homotransplantability (*8*).

The MC1A tumor shows the highest degree of homotransplantability of the 3 tumors tested. We have shown that it also evokes the greatest immune response. It follows that MC1A should reveal the best capacity to resist transplantation immunity. In fact, this high resistance could not only be deduced from the points considered previously (*i.e.*, the existence of the highest antibody response and the highest transplantability), but could also be demonstrated by direct transplantation tests. We have shown that a second intraperitoneal graft of MC1M cannot withstand the immunizing effect of a primary intraperitoneal MC1M graft. But MC1A grafted intraperitoneally after MC1M overcomes the immunity produced by the first graft. Similar data (unpublished) were obtained with a primary inoculation of 6C3HED. The MC1M tumor inoculated 7 days after an intraperitoneal graft of 6C3HED does not grow progressively, whereas MC1A can grow progressively under similar conditions.

Immunizing Effects of Successful Homografts and Their Significance for the Phenomenon of Secondary Response

The immunizing effects of homotransplantable tumors were tested by challenging the host with a second test graft after removal of the first. A primary graft of MC1M or MC1A conferred immunity, which prevented the growth of a second. According to the general theory that ". . . the shorter the survival time the more violent is the reaction. . . ." (*25*) one should relate the shorter survival time of the second graft to a "more violent" reaction, *i.e.*, an increased antibody response brought about by the second graft. With skin grafts, it has been shown that a second transplant, with a shorter survival time, raises the hosts' immune response, as measured by agglutinin titer (*34*). In this instance the shorter survival time reflects a secondary response.

But in the case of the tumors, agglutinin determinations after 1 MC1A transplant were found to be identical to those following a second MC1A transplant. It was concluded that the second graft was destroyed by the antibodies produced by the first. It appears, therefore, that a shortening in the survival time of tumor homografts does not by itself indicate

that heightened host resistance was produced by a secondary response. In our study it has indicated only that a condition of decreased tumor resistance was produced by exposing a tumor, in its lag phase of growth, to a preformed immune environment.

Second grafts, after a primary transplant of 6C3HED and, to a lesser extent, of MC1M, have produced a secondary response of agglutinins. Since an interval of 14 days between the 2 transplants caused a stronger response, both in agglutinin titers and cytotoxic effects, than a 7-day interval, it seems that a secondary response is achieved if the second antigenic stimulus acts on the lymph nodes when the primary response is already in its descending phase (or at least past the ascending phase). If the time interval is too short, the second stimulus is probably incorporated with the first without any increase in titer, in the same way that a larger inoculum does not increase the immune response. Thus, up to a certain time limit, the greater the time interval between 2 inoculations, the greater the immune response. The results with leukocyte titers found for skin grafts (*14*) may also suggest the effect of a time interval on the production of increased immunity.

The lack of a secondary response in 2 tumor grafts made at short intervals may explain the fact that cell inoculums larger than 15×10^6 did not yield a higher agglutinin titer in homotransplantable tumors. The 30×10^6 cell inoculum may be regarded as two 15×10^6 inoculums given at a time interval which, being zero, was too small to result in increased response. The lack of dose effect when tumors are inoculated into the same lymphatic "basin" appears at first to differ from the result obtained with skin grafts (*22,23,25*) in which ". . . the larger the amount grafted, the shorter is the expectation of life . . . ." (*25*). The reason for the apparent contradiction may be that the survival time of tissues is determined not merely by the intensity of antibody response but also by the length of time between grafting and the inception of antibody production. We have some evidence (unpublished data) that larger inoculums result in a shorter induction period, *i.e.*, an earlier rise of the functional antibody production. Whether or not this may affect the survival of tissue grafts is a matter for further investigation.

Decreased Antigenicity as a Possible Basis for Homotransplantability

Although it has been clearly shown in the present study that the more homotransplantable tumors are also more antigenic, the possibility cannot be ruled out that another mechanism of homotransplantability, based on decreased antigenicity, may characterize other tumors. This possibility has been discussed as a general concept to account for homotransplantability (*3–5,9*), but the experimental data which have been presented to verify this hypothesis (*9,17*) do not as yet seem to us to be conclusive.

Two of the 3 tumors studied by Hauschka *et al.* and by Amos with serologic methods (*9,17*) do not show the phenomenon to be explained, that is, the strain A lymphoma #1 (*5*) does not have a high degree of homotransplantability and this is true to an even greater degree for the

DBA-adapted 6C3HED lymphosarcoma (*9*). Amos has considered this point and has stated, "The fact that polyploid tumors are not entirely indiscriminate in their histocompatibility requirements (*i.e.*, L. No. 1, 6C3HED in DBA) may be due in part to metabolic differences between graft and host." (*17*). As for the DBA-adapted 6C3HED, it is known that other C3H tumors can grow progressively in DBA mice. These assumed metabolic differences in the tetraploid 6C3HED cannot, therefore, be due to some properties specific to C3H. They would have to be due to specific properties of the graft, *i.e.*, some specific requirements of these particular tumors. It seems to us more likely that the incompatibility in a wide range of foreign hosts derives simply from the capacity of the tetraploid 6C3HED to produce an immune response in foreign strains and that this also applies to strain A lymphoma #1. It would also be of interest to know whether the unabsorbed titers obtained for these 2 tumors (*17*) involve a secondary response.

Carcinoma TA32 was the only tumor that exhibited a correlation between decreased antigenicity, as measured by *in vitro* absorption of hemagglutinins, and a high degree of homotransplantability (*17*). Only when a direct test of the immunizing effect of this tumor reveals low antigenicity will it be shown that homotransplantability can be produced by 2 distinct mechanisms.

Summary

Using the three ascites tumors 6C3HED, MC1M, and MC1A, we studied the antibody response elicited by tumors with different degrees of homotransplantability. The antibody response was measured by hemagglutinin determinations and transplantation tests.

The intensity of hemagglutinin production paralleled the intensity of immunity, as measured by the degree of host resistance to tumor grafts.

It has been found for the 3 tumors that the higher the degree of homotransplantability, the more intense was the immune response evoked by the homograft. It is, therefore, concluded that the loss of strain specificity is due to an increase in the capacity to resist isoimmune response.

An experimental analysis of the immunizing effects of the 3 tumors has shown that the more antigenic the tumor, the more resistant it was to antibody response.

It has been demonstrated that there is an *in vivo* absorption of antibodies by the progressively growing homografts and that the rate of absorption is correlated with the degree of homotransplantability. If we assume that this absorption is carried out by extracellular antigen, the mechanism of tumor resistance to antibody response can then be explained by extracellular neutralization of antibodies.

Evidence has been presented that regression of a second graft of a homotransplantable tumor in a host previously inoculated with a homotransplantable-tumor graft that was removed cannot be attributed to a secondary response elicited by the second transplant. The growth of the second graft is prevented by the antibodies produced by the growth

of the first. The antibody response produced by 2 successive tumor inoculations has been discussed in relation to the phenomenon of secondary response.

In the 3 tumors tested common antigens are active in eliciting transplantation immunity. This has also been confirmed by *in vitro* absorption tests for hemagglutinins.

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Biological Testing of Sulfur Isosteres of Carcinogenic Hydrocarbons[1,2]

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Experimental production of cancer by application of crude chemicals like coal tar was first achieved during the early years of the present century (*1–3*). The specific cancer-producing fractions were later isolated and identified in pure form. The introduction of these chemical compounds as a consistently reproducible means of inducing malignant tumors in animals is one of the most valuable contributions that chemists have made to the study of cancer. They have further attempted to study the problem of the structural features underlying carcinogenic action in polycyclic hydrocarbons (*4,5*). Extensive research in this field, however, has not yielded a general principle to explain the mechanism of action of carcinogenic substances and the relationship of carcinogenesis to chemical structure.

In 1946, Robinson (*6*) suggested a novel scheme to explain carcinogenic activity of polycyclic hydrocarbons. According to him the essential feature of carcinogenicity in most of the polycyclic hydrocarbons is the 9,10 double bond of phenanthrene (called the K region), which is activated by suitable substitution or by additional benzene rings. He presumed the possibility of reaction at an activated phenanthrene bridge, and on the basis of this hypothesis Tilak (*7*) undertook synthesis of hydrocarbons with replacement of the benzene ring by the isosteric thiophene nucleus. Recently, Ghaisas, Rabindran, and Tilak (*8*) and Rabindran and Tilak (*9,10*) have synthesized several thiophene isosteres (thiosteres) of carcinogenic hydrocarbons. The present investigation was planned to test the biological activity of these polycyclic compounds, in which the phenanthrene bridge is substituted by the isosteric thiophene ring.

Materials and Methods

The 7 thiosteres of 5 known carcinogens investigated for their carcinogenic activity are shown in text-figure 1.

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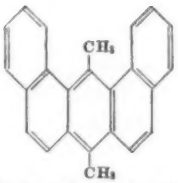
[2] The investigations are being carried out under a program financed by the Council of Scientific and Industrial Research, New Delhi, India.

[3] We are greatly indebted to Prof. B. D. Tilak for supplying the sulfur analogues of polycyclic hydrocarbons synthesized by him at the Department of Chemical Technology, University of Bombay.

Grateful thanks are due to Dr. V. R. Khanolkar and Dr. T. B. Panse for their encouragement and valuable advice during the course of the studies.

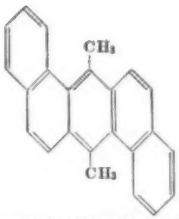
TEXT-FIGURE 1.—Polycyclic compounds and their thiosteres.

I



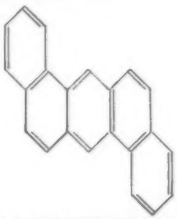
9,10-Dimethyl-1,2,7,8-dibenzanthracene

II



9,10-Dimethyl-1,2,5,6-dibenzanthracene

III

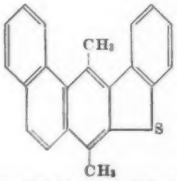


1,2,5,6-Dibenzanthracene

IV

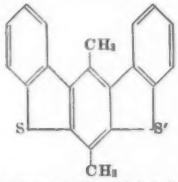
3,4-Benzophenanthrene

No. 1



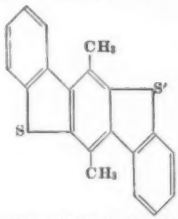
4,9-Dimethyl-2,3,5,6-dibenzothiophanthrene

No. 2



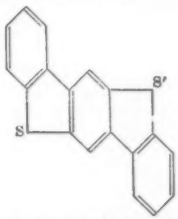
6,12-Dimethylbenzo(1,2-b,5,4-b′)dithionaphthene

No. 3



6,12-Dimethylbenzo(1,2-b,4,5-b′)dithionaphthene

No. 4

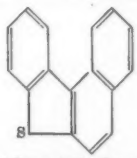


Benzo(1,2-b,4,5-b′)dithionaphthene

No. 5

Thionaphtheno-4,5,4′,5′-thionaphthene

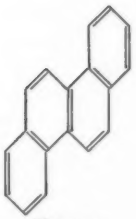
No. 6



3,4-Benzo-9-thiafluorene

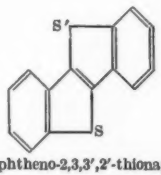
TEXT-FIGURE 1.—Continued.

V



Chrysene

No. 7



Thionaphtheno-2,3,3',2'-thionaphthene

The compounds under investigation will be referred to in the text by their respective numbers in the preceding text-figure.

Swiss strain mice originally brought from the Virus Research Centre, Poona, and bred by brother × sister mating in this laboratory were used in the experiment. All the experimental animals were 3-month-old virgin females. Two standard methods were used for testing the compounds: 1) skin painting; 2) subcutaneous injection.

1) Skin painting.—The skin on the back of the mouse was carefully clipped, 1 day before application of the solution. A 0.5 percent solution of the substance in pure benzene (thiophene-free) was administered twice a week by a remote-pipette device, 1 drop of the solution being dropped on the clipped area of the skin. The paintings were continued until the appearance of tumor and, in the case of inactive substances, until the death of the animal.

2) Subcutaneous injection.—Each animal was given a single subcutaneous injection of 2 mg. of the substance dissolved in 0.2 ml. of tricaprylin. Each experimental group consisted of 12 females, except in the case of compound no. 1, where 14 females were used for painting and 3 females for injection, because sufficient quantity of the compound was not available for injection.

All the animals were sacrificed when they appeared weak and emaciated. The tumor tissue was fixed in Bouin's fixative for histopathologic study. Gross mounts of mammary glands and sections of ovary and adrenal were prepared in order to study the effect of the test compound on these organs, the results of which will be reported elsewhere.

Experimental Results

Table 1 presents results of biological testing of 7 sulfur isoteres of carcinogenic hydrocarbons.

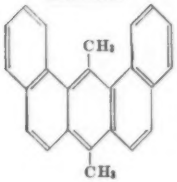
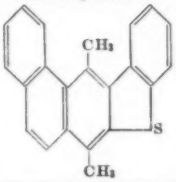
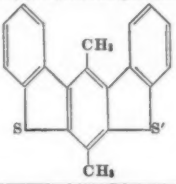
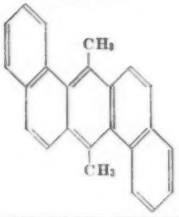
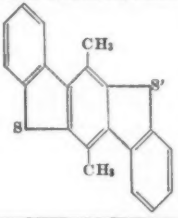
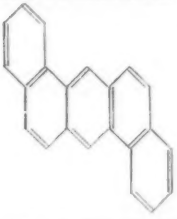
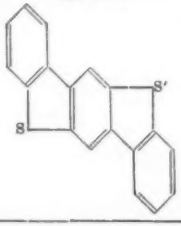
The first 4 compounds in the series belong to the group of dibenzanthracenes.

Compound No. 1: Thiostere of 9,10-Dimethyl-1,2,7,8-dibenzanthracene

Painted group.—The majority of mice, 11 of 14 painted with compound no. 1, developed epidermoid carcinoma at the site of painting after 8 months. Three animals died early with papillomatous growth at the site

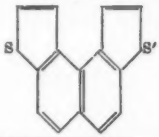
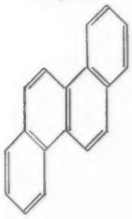
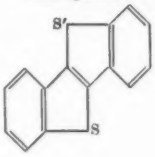
of painting. Solid tumor was first palpated after 4 months' treatment. The tumor growth varied, generally beginning as papilloma, and progressed at a slow rate. If it was maintained for a long time it ulcerated centrally and infiltrated the surrounding tissue (fig. 1). On histologic examination, marked keratinization and extensive formation of large and small epithelial pearls were seen. Around these pearls were irregular

TABLE 1.—*Biological testing of thiosteres of polycyclic hydrocarbons*

Parent hydrocarbons	Test compounds (*thiosteres*)	No. of animals*	Duration of the experiment (*months*)	No. animals with tumor (*tumor type*)
9,10-Dimethyl-1,2,7,8-dibenzanthracene 	(1) 4,9-Dimethyl-2,3,5,6-dibenzothiophanthrene 	14	8–10	11 (epidermoid carcinoma)
		5	4–6	2 (fibrosarcoma)
	(2) 6,12-Dimethylbenzo(1, 2-b,5,4-b')dithionaphthene 	12	19–22	1 (epidermoid carcinoma); 1 (fibrosarcoma); 1 (papilloma)
		13	4–6	12 (fibrosarcoma)
9,10-Dimethyl-1,2,5,6-dibenzanthracene 	(3) 6,12-Dimethylbenzo(1, 2-b,4,5-b')dithionaphthene 	*12*	6	9 (fibrosarcoma)
1,2,5,6-Dibenzanthracene 	(4) Benzo(1,2-b,4,5-b')dithionaphthene 	*12*	6	None

*Figures in italics: number of animals given 2 mg. of compound in 2 ml. tricaprylin subcutaneously. The others received 0.5 percent of compound in benzene painted on the skin.

TABLE 1.—*Biological testing of thiosteres of polycyclic hydrocarbons*—Continued

Parent hydrocarbons	Test compounds (*thiosteres*)	No. of animals*	Duration of the experiment (*months*)	No. animals with tumor (*tumor type*)
3,4-Benzophenanthrene	(5) Thionaphtheno-4,5,4',5'-thionaphthene 	12	15-17	None
		12	18-22	None
	(6) 3,4-Benzo-9-thiaflourene	12	16-20	None
		12	18-22	None
Chrysene 	(7) Thionaphtheno-2,3,3',2'-thionaphthene 	12	17-19	None
		12	15-24	None

clumps of polyhedral cells with large, often pyknotic nuclei. The cytoplasm was abundant; it was acidophilic and often contained coarse keratohyalin granules. Mitoses were frequent in these smaller cells (figs. 2 and 3). Animals treated with this compound had definite enlargement of spleen and lymph nodes. This condition was seen in 11 of 14 females.

Injected group.—Of the 3 injected animals, 2 developed fibrosarcomas at the site of injection and 1 died at an early age.

Compound No. 2: Thiostere of 9,10-Dimethyl-1,2,7,8-dibenzanthracene

Painted group.—Skin painting with this compound was continued for 19 months; only 1 mouse developed epidermoid carcinoma after 11 months of painting, another developed a fibrosarcoma 1.4 cm. in diameter, while 1 had 2 slow-growing papillomas about the size of a pinhead.

Injected group.—Twelve of 13 females given this compound developed fibrosarcoma at the site of injection within 4 to 6 months of injection. On gross examination the tumors appeared as a compact mass with a smooth, rounded white surface (fig. 4). Microscopically, the tumors showed closely packed spindle-shaped cells, which varied in size from medium to large and in shape from blunt to long, narrow spindle cells. The cytoplasm was pale and eosinophilic, the nuclei were elongated and

hypochromatic and were located centrally in the cells. Between the nucleated cells there were many smaller non-nucleated fragments. These represented the tapered ends of long cells cut at such an angle that the nuclei were not included. Mitotic figures were usually abundant and invariably exhibited irregular divisions (figs. 5 and 6).

Compound No. 3: Thiostere of 9,10-Dimethyl-1,2,5,6-dibenzanthracene

This compound was tested only by injection, because sufficient quantity for continuous painting was not available. One subcutaneous injection of the substance in tricaprylin was given to each of a group of 12 female mice. Nine of 12 females developed fibrosarcomas within 4 to 6 months after injection. Three females are still under observation.

Compound No. 4: Thiostere of 1,2,5,6-Dibenzanthracene

This compound was also tested only by subcutaneous injection. All 12 females developed small cysts at the site of injection, which regressed after some time. Seven of these animals looked weak and had to be sacrificed after 6 months of injection. No malignant growth of the subcutis was observed in these mice. Five females are still under observation.

Compound No. 5: Thiostere of 3,4-Benzophenanthrene

Painted group.—The animals were painted with compound no. 5 for 15 to 17 months and there was no tumor development at the site of painting.

Injected group.—No palpable tumor at the site of injection was seen in this group of mice; they were observed up to the age of 18 to 22 months, at which time they were sacrificed.

Compound No. 6: Thiostere of 3,4-Benzophenanthrene

Painted group.—The skin paintings were continued for 16 to 20 months. There was no development of tumor at the site of painting.

Injected group.—The group of 12 females given this compound developed no tumors at the site of injection up to the age of 18 to 22 months, when they were sacrificed.

Compound No. 7: Thiostere of Chrysene

Painted group.—Twelve female mice were painted with the compound for 17 to 19 months. There was no tumor development at the site of painting.

Injected group.—The group that received 1 subcutaneous injection of this compound in tricaprylin developed no tumors at the site of injection until the age of 15 to 24 months.

Discussion

Robinson has related the carcinogenic activity of polycyclic hydrocarbons to the presence of an activated phenanthrene bridge (*6*). Thiosteres of carcinogenic hydrocarbons were therefore synthesized with a

view to studying the significance of the phenanthrene bridge in chemical carcinogenesis (*7–10*). Biological testing of 7 such thiosteres has been reported here.

9,10-Dimethyl-1,2,7,8-dibenzanthracene, a *meso*-dimethyl derivative of 1,2,7,8-dibenzanthracene is a confirmed carcinogen. There are 2 reactive centers in this carcinogen—the 2 phenanthrene double bonds. Substitution of 1 of the phenanthrene bridges by a sulfur atom leads to the thiostere 4,9-dimethyl-2,3,5,6-dibenzothiophanthrene (compound no. 1), which proved highly carcinogenic due to the increased activation of the remaining active center. The results of biological tests of this benzo-derivative, reported here, show significantly high carcinogenic activity when painted on mouse skin as well as after subcutaneous injection. The majority of the animals in both experimental groups developed tumors at the site of administration. These results confirm previous findings (*11*) and support Robinson's hypothesis of an activated phenanthrene bridge.

Compound no.2 (*8*) is another thiophene isostere of 9,10-dimethyl-1,2,7,8-dibenzanthracene, in which both the phenanthrene double bonds have been removed and the *meso*- positions have been blocked by methyl groups. This is a key compound which should prove inactive in the absence of an active phenanthrene bridge. However, results of biological tests of this compound indicate weak carcinogenic activity in skin painting and very strong activity with subcutaneous injection. Within a comparatively short latent period of 12 weeks, 12 of 13 experimental animals developed frank fibrosarcomas.

In compound no. 3, both the K regions in the weak carcinogen 9,10-dimethyl-1,2,5,6-dibenzanthracene have been replaced by isosteric substitution with 2 thiophene rings. Compound no. 3 is therefore a key compound, like compound no. 2. As in the case of compound no. 2 this compound was also expected to be inactive; however, a fair degree of carcinogenic activity has been found with subcutaneous injection—9 of 12 mice developed fibrosarcomas within 6 months of treatment. The carcinogenicity of key compounds nos. 2 and 3 are thus outstanding exceptions to the K-region hypothesis.

In compound no. 4, both the K regions in the weakly carcinogenic hydrocarbon 1,2,5,6-dibenzanthracene have been replaced by isosteric substitution with 2 thiophene rings. The compound has been under test by subcutaneous injection for the last 6 months. No tumors have appeared as yet and it is likely that the compound is inactive—a conclusion which would support the K-region hypothesis.

Biological testing of 4 dibenzanthracenes thus presents 2 exceptions to the K-region hypothesis (compounds nos. 2 and 3). It may be noted that all 3 active compounds in the group have their *meso*- positions blocked by methyl groups.

The other important known carcinogen is 3,4-benzophenanthrene, of which compounds nos. 5 and 6 are thiosteres. According to Robinson's

hypothesis, discussed by Tilak (7), isosteric replacement of rings A and D in 3,4-benzophenanthrene, as in compound no. 5, should not affect its carcinogenic activity, whereas replacement of ring B or C, as in compound no. 6, should only give a more potent carcinogen, since 1 of the 2 competing reactive K regions is replaced. Both compounds nos. 5 and 6 were thus expected to be carcinogenic. However, thorough investigations on both these compounds, for sufficiently long duration, have not shown any carcinogenic activity at the site of administration, either the skin or subcutis. The results are thus totally contrary to Robinson's hypothesis. A key compound in this series would be a dithiopheno analogue, where both rings B and C are replaced by thiophene. This compound (which may be expected to be inactive according to the K-region hypothesis) has recently been synthesized by Tilak and will soon be tested.

In compound no. 7 both the K-region bonds of the weakly active parent hydrocarbon chrysene have been replaced by sulfur atoms, and the subsequent inactivity of the substance, recorded in the table, supports the K-region hypothesis.

In summary, it may be seen that the results for 4 test compounds (nos. 2, 3, 5, and 6) of a total of 7, cannot be explained by the K-region hypothesis. Evaluation of these findings proves that the 9,10 double bond of phenanthrene (K region) alone is not the determining factor for carcinogenicity of polycyclic hydrocarbons. French chemists, notably Pullman and Pullman (*12*), have carried out extensive quantitative investigations on the electronic distribution in and near the K region and established an "electrical index" for this region, which, if it exceeds the threshold value, would render the molecule carcinogenic. According to them a high bond order in the K region and certain other factors which are not yet clearly understood lead to carcinogenesis. Besides the active K region, reactivity at other positions, particularly the *meso*- positions (the L region) must also be considered in determining the relationship between chemical structure and carcinogenicity. An outstanding example among the thiosteres is the inactivity of compound no. 4 as compared with the activity of compounds nos. 3 and 2.

The mode of action of carcinogens and their metabolism in the body are other complex problems that need investigation. In addition to the action of a test substance at the site of administration it is important to study its effect on the general metabolism of different organs. Many known carcinogens and chemically allied compounds are known to exhibit hormone-mimetic action. A complete study of cancer-susceptible organs, like mammary glands, ovaries, and adrenals, of treated mice has therefore been undertaken.

Compounds nos. 4, 5, 6, and 7, which have proved specifically inactive on the skin and subcutis, have induced a large number of ovarian cysts and tumors. The histology of these lesions and the etiology might throw some light on the mode of action of these inactive thiosteres of carcinogenic hydrocarbons.

Summary

Seven sulfur isosteres (thiosteres) of carcinogenic hydrocarbons have been tested for their carcinogenicity on Swiss mice by 2 standard techniques: *a*) skin painting; *b*) subcutaneous injection.

Biological testing of these compounds was undertaken to investigate the significance of the activated phenanthrene bridge in chemical carcinogenesis.

The observations on carcinogenicity have been reported and discussed with reference to the chemical structure of these compounds. The findings on 4 compounds—6,12-dimethylbenzo(1,2-b,5,4-b′)dithionaphthene, thiostere of 9,10-dimethyl-1,2,7,8-dibenzanthracene; 6,12-dimethylbenzo-(1,2-b,4,5-b′)dithionaphthene, thiostere of 9,10-dimethyl-1,2,5,6-dibenzanthracene; thionaphtheno-4,5,4′,5′-thionaphthene; 3,4-benzo-9-thiafluorene, thiostere of 3,4-benzophenanthrene—do not support the hypothesis of an activated phenanthrene bridge that is related to carcinogenicity.

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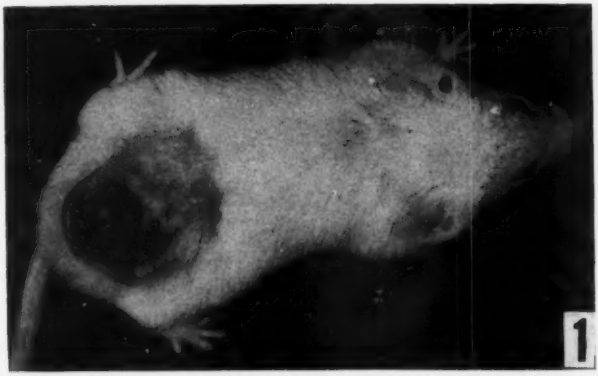
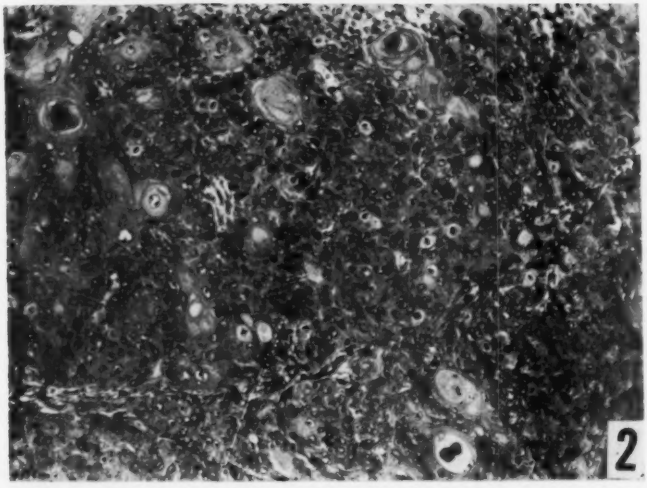
PLATE 36

FIGURE 1.—Skin tumor induced in a Swiss female by painting with 0.5 percent 4,9-dimethyl-2,3,5,6-dibenzothiophanthrene in benzene.

FIGURE 2.—Histologic picture of epidermoid carcinoma induced by 4,9-dimethyl-2,3,5,6-dibenzothiophanthrene. × 150

FIGURE 3.—A portion of the same epidermoid carcinoma shown in figure 2 under higher magnification, showing pearl formation. × 300





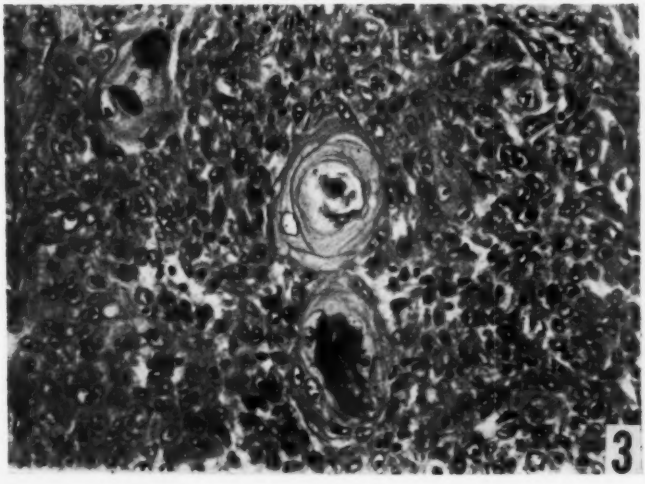


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PLATE 37

FIGURE 4.—Fibrosarcoma of the subcutis induced in a Swiss female by subcutaneous injection of 6,12-dimethylbenzo(1,2-b,5,4-b′)dithionaphthene—2 mg. in 0.2 ml. of tricaprylin.

FIGURE 5.—Microscopic picture of a fibrosarcoma. × 150

FIGURE 6.—A portion of a fibrosarcoma under high magnification. × 300

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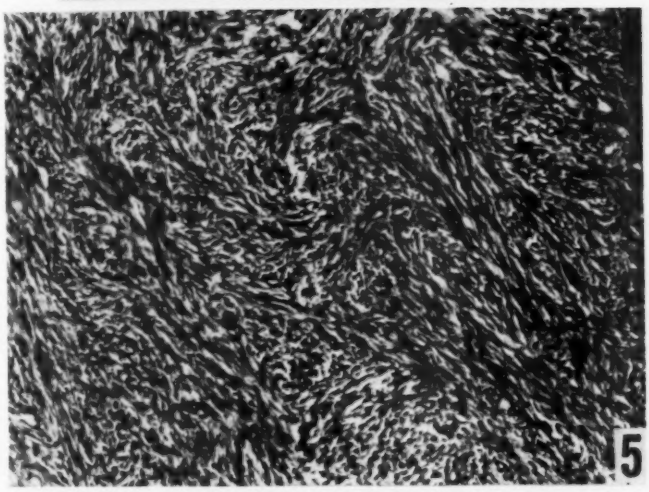
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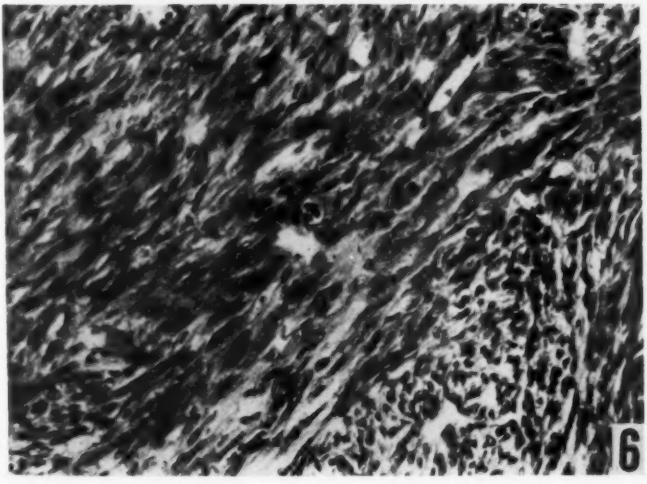
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Deoxyribosidic Compounds in the Novikoff Rat Hepatoma[1, 2]

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The increasing body of evidence demonstrating the unique role played by deoxyribonucleic acid (DNA) in cell division, as an essential component of the chromosomes, and in heredity, as the genic material, has led to a great deal of interest in the biosynthesis of this material. We are approaching the question of DNA synthesis by determining whether deoxyribosidic compounds other than DNA exist in normal and cancerous tissues, by isolating and identifying such compounds, and by establishing the role of these compounds as precursors of DNA.

In a previous paper (*2*) we were able to show that deoxyribosidic compounds other than DNA were widely distributed in normal and cancerous tissues and that the concentration of these compounds in mouse tumors was considerably higher than in most normal mouse tissues. In a rat hepatoma, however, the concentration of deoxyribosidic compounds was found to be about the same as in normal rat liver. In the present paper, studies on the latter 2 tissues have been extended to the isolation and identification of the deoxyribosides. The results show that, although only a single deoxyriboside was present in rat liver, 2 additional deoxyribosides and unidentified deoxynucleotide-like compounds were present in the hepatoma.

Material and Methods

Tissue collection.—Normal and tumor tissues were obtained from rats under ether anesthesia, frozen immediately in liquid nitrogen, and stored at −20° C. The Novikoff hepatoma was maintained by intraperitoneal transplantation and the solid growth was harvested once a week.[4]

Preparation of tissue extracts.—In the experiments to be reported in this paper, 75 to 100 gm. lots of frozen tissue were homogenized in the Waring blendor with 3 to 4 volumes of ice-cold 12 percent trichloroacetic acid (TCA). The insoluble material was removed by centrifugation for 30

[1] Received for publication December 13, 1956.

[2] A preliminary report of some of the material presented in this paper appeared previously (*1*).

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] The author is greatly indebted to Mrs. Leona W. Brownell for a supply of this tumor and for many of the microbiological assays necessary in these experiments.

minutes at 5000 r.p.m. and 0 to 2° C. The supernatant fluid was filtered and extracted 4 times with an equal volume of ether to remove the TCA. After the final extraction, the solution was neutralized to *p*H 7. The neutral solution was chromatographed as such or was concentrated by evaporation at reduced pressure before chromatography (*2*).

Column chromatography.—Amberlite XE-64-H^+ resin was prepared for chromatography by pouring a 15 × 2 cm. column and washing the column with 3 to 4 bed volumes of 0.1 *N* HCl and then with water, until the effluent was no longer acid.

Dowex-1-formate columns (15 × 2 cm.) were prepared as described by Hurlbert *et al.* (*3*). In the case of Dowex-1 columns in which buffers were used, the columns were prepared in a similar manner but were equilibrated with 0.01 *N* formic acid adjusted to *p*H 9.5 with NH_4OH before applying the sample, dissolved in the same buffer.

The progress of chromatography was followed by determining the optical density of the effluent at 260 mμ in the Beckman Model DU spectrophotometer. After the sample was applied, the column was washed with solvent until the optical density reached the base-line value before changing to a new eluent.

Paper chromatography.—The following solvent systems were used:

Butanol-H_2O: butanol saturated with distilled water, NH_3 atmosphere (*4*).
Butanol-H_2O-acetic acid: 5 ml. of acetic acid in 25 ml. butanol saturated with water (*5*).
Butanol-H_3BO_3: butanol saturated with 5 percent aqueous boric-acid solution (*6*).
H_2O, *p*H 10: distilled water adjusted to *p*H 10 with dilute NH_4OH (*7*).
Isopropanol-H_2O: 85 ml. isopropanol, 15 ml. H_2O, 1.3 ml. concentrated NH_4OH (*7*).
Isopropanol-H_2O-HCl: 170 ml. isopropanol, 41 ml. concentrated HCl, water to make 250 ml. (*7*).

Solutions to be chromatographed were applied as bands, 1 cm. wide and 7 cm. from the end of the Whatman #40 paper, and were dried with warm air. Descending chromatography was used. After each chromatogram, the position of the deoxyribosidic compounds was determined microbiologically. The deoxyribosidic compounds were eluted with water and applied to a narrower sheet of paper before rechromatography with other solvents. In the final stages of purification of each compound, the tissue deoxynucleosides were applied as 1 cm. spots and their position on the chromatogram was detected by use of an SL Mineralite SV-41 lamp, as well as by microbiological assay.

Microbiological assay.—The amount of deoxyribosidic material in samples obtained during column chromatography or chromatography on paper was determined microbiologically with the organism *Lactobacillus acidophilus* R-26 (ATCC #11506). This organism specifically requires deoxyribosidic compounds for growth. Equimolar amounts of any of the 5 deoxynucleosides or the 4 deoxynucleotides tested gave the

same amount of growth (*2,8*). The amount of growth observed was linear in the range 0.1 to 0.4 μg. of thymidine, or its equivalent, in a total volume of 3.0 ml. Growth was measured turbidimetrically (*2*) and the culture medium used was a modification of the one used before.[5]

Other methods.—The cysteine-sulfuric acid reaction for deoxyribose was carried out as described by Brody (*9*). Pure deoxycytidine, deoxyuridine, or thymidine were used as standards.[6] The ultraviolet spectra of eluates of paper chromatograms were determined in 1 cm. quartz cuvettes in the Beckman Model DU spectrophotometer. Blanks consisted of eluates made from paper strips that had the same dimensions and occupied the same position as the sample on control chromatograms. The spectral characteristics of the pure deoxynucleosides were determined after elution from similar chromatograms and were also corrected with appropriate filter-paper blanks. Total nitrogen was determined by Kjeldahl digestion and nesslerization (*10*). Ammonia nitrogen was removed prior to digestion by treating aliquots of the solution *in vacuo* with alkali and absorbing the ammonia released in sulfuric acid. Solutions were concentrated either by lyophilization or by use of a rotating evaporator (*2*).

Results

In a previous paper (*2*) we reported the isolation of deoxycytidine from normal rat blood and of deoxycytidine and deoxyuridine from rat liver. The experiments reported in this paper indicate that the deoxyuridine isolated previously from liver was an artefact, probably produced by deamination of deoxycytidine during the many paper-chromatographic separations required for the isolations. The following experiments, in which simpler isolation procedures were developed, indicate that deoxycytidine was the main deoxyribosidic compound in rat-liver extracts.

Behavior of Pure Pyrimidine Deoxynucleosides on Ion-Exchange Columns

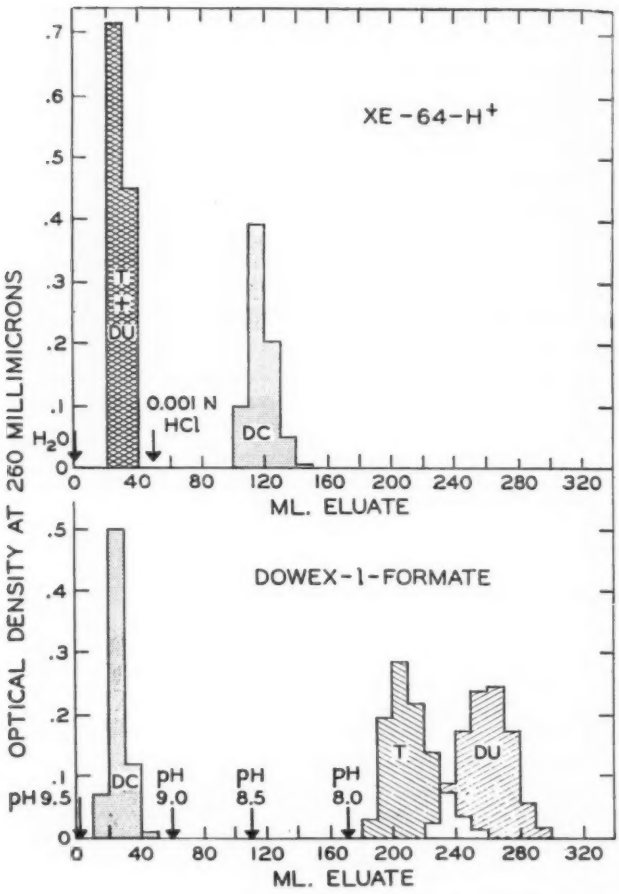
As shown in text-figure 1, pure deoxycytidine could be separated readily from pure deoxyuridine and thymidine in 2 ways. Deoxycytidine was adsorbed on XE-64-H^+ resin, while the other 2 nucleosides were adsorbed on Dowex-1 at *p*H 9.5. It will be noted that, although some separation of thymidine and deoxyuridine occurred in the Dowex-1 chromatogram, the separation was incomplete.

Isolation and Identification of Normal Rat-Liver Deoxynucleosides

When a rat-liver extract neutralized to *p*H 7 was passed through an XE-64-H^+ column, about 10 percent of the deoxyribosidic activity passed through the column immediately. The deoxyribosidic material that had been adsorbed from the liver extract was not eluted from the resin column

[5] E. Hoff-Jorgensen, personal communication.

[6] The pure deoxynucleosides used as standards were the commercial products used previously (*2*).



TEXT-FIGURE 1.—Chromatography of pure pyrimidine deoxynucleosides on ion-exchange columns. Mixtures of thymidine (T), deoxycytidine (DC), and deoxyuridine (DU) were chromatographed on 16 × 1.0 cm. XE-64-H+ columns or on 15 × 1.0 cm. Dowex-1-formate columns. In the former, the samples were dissolved in water, while in the latter the deoxynucleosides were dissolved in 0.01 *N* formic acid adjusted to *p*H 9.5 with NH_4OH. Collection of samples and elution as indicated.

when the latter was washed with 0.001 *N* HCl, as had been the case with pure deoxycytidine (text-fig. 1), but appeared in the eluate only when the HCl concentration was increased to 0.01 *N*. Nevertheless, when the latter eluate was concentrated and chromatographed on Whatman #40 paper, using the solvents listed in table 1, the deoxyribosidic material migrated at the same rate as pure deoxycytidine. In another experiment, the liver deoxynucleoside isolated from the water chromatogram (table 1) was mixed with either pure deoxycytidine or pure deoxyuridine and re-

chromatographed. The positions of the liver deoxynucleoside, pure deoxycytidine, or a mixture of the 2 were identical and indicated that the liver deoxyriboside was deoxycytidine. Further support for this conclusion was obtained from the ultraviolet spectrum of the isolated material compared with that of pure deoxycytidine (table 2). The wavelengths of maximum and minimum absorption of the liver compound were very nearly the same as those of pure deoxycytidine and the optical-density ratios of the isolated compound also approached those of pure deoxycytidine. As a final test of the correctness of the conclusion, the amount of deoxycytidine present in the final liver eluate, table 1, was determined by 3 independent methods: (1) microbiological assay, (2) the color produced in the cysteine-sulfuric acid reaction for deoxyribose, and (3) the intensity of absorption at 280 mμ in acid solution, using pure deoxycytidine as the standard. The results were as follows:

Method of measurement	*Deoxycytidine isolated from 100 gm. liver (μg.)*
Microbiological assay	295
Cysteine-sulfuric acid reaction	301
Ultraviolet absorption	296

The amount of nitrogen present in this eluate was 78.3 μg. as compared to 41.2 μg. calculated from the average deoxycytidine content. Excess nitrogen in similar amounts has been observed in 3 other liver deoxycytidine preparations. We cannot explain this discrepancy at present.

TABLE 1.—*Paper chromatography of 0.01 N HCl eluate obtained from chromatography of rat-liver extract on XE-64-H+**

Material chromatographed	R_f† after chromatography with:		
	Butanol-H_2O and butanol-H_2O-acetic	H_2O, *p*H 10	Butanol-H_3BO_3
Eluate	0. 36	0. 78	0. 26
Pure deoxycytidine	0. 37	0. 76	0. 26
Pure deoxyuridine	0. 52	—	0. 40
Pure thymidine	0. 65	—	0. 55

*The solvents listed were used consecutively, with reisolation of the deoxyribosidic material after each chromatogram. The position of the spots was determined microbiologically.

†R_f = ratio of the average distance traveled by the spot to that traveled by the solvent front.

TABLE 2.—*Spectral characteristics of pure and liver deoxycytidine**

Material	Absorption maximum (*mμ*)	Absorption minimum (*mμ*)	Optical-density ratios†	
			$\frac{280}{260}$	$\frac{250}{260}$
Liver deoxyriboside	278–280	240–241	1. 93	0. 48
Pure deoxycytidine	280	241	2. 04	0. 44

*The deoxyribosidic material was eluted from the final chromatogram in butanol-H_3BO_3 (table 1), and the spectral characteristics were determined in 0.025 *N* HCl.

†Ratios of the optical densities at the wavelengths indicated.

Other experiments, involving use of Dowex-1-resin columns and offering additional support for the conclusion that the main, if not the sole, deoxyribosidic compound present in rat-liver extracts was deoxycytidine, should also be described. In a previous paper (*2*) we reported that when rat-liver extracts at *p*H 7 were passed through Dowex-1 columns followed by water, 82 percent of the deoxyribosidic compounds was recovered in the effluent. In more recent experiments in which elution of the columns with water was continued until the optical density of the effluent at 260 mμ had returned to base-line levels, an average of 92 percent of the deoxyribosidic material was recovered in the eluate (*11*). Since nucleosides are not adsorbed by Dowex-1 under these conditions (*see* text-fig. 1), while nucleotides are, the results indicated that over 90 percent of the liver deoxyribosidic compounds were nucleosides. When this nucleoside fraction was adjusted to *p*H 9.5 and passed through a Dowex-1 column, as in text-figure 1, all the deoxyribosidic activity appeared in the *p*H 9.5 eluate (deoxycytidine fraction) and none in the *p*H 8 eluate (thymidine-deoxyuridine fraction).

It should be noted that the amount of deoxycytidine isolated from the liver was considerably less than would be predicted (1) from the content of deoxyribosidic compounds in this tissue (*2,11*) and (2) from the conclusion that deoxycytidine was the main deoxyribosidic compound present. The low yields in this experiment, and also in the isolations to be reported for the hepatoma, were due to incomplete extraction of the tissue and to the inevitable losses from sampling and manipulation during isolation.

Isolation and Identification of Hepatoma Deoxynucleosides

When a neutral extract of the Novikoff hepatoma was chromatographed on an XE-64-H^+ column, 2 deoxyribosidic fractions were obtained. The A fraction contained about 50 percent of the microbiological activity and came off the column when washed with water, while the B or deoxycytidine fraction contained the remaining activity and was eluted with dilute HCl. The A and B eluates were concentrated and chromatographed separately on paper with the solvents indicated in table 3. The results show that the A fraction contained 2 components, while the B fraction contained a single deoxyribosidic component. On the basis of migration rate, the slower A component appeared to correspond to deoxyuridine, while the faster A component migrated at the same rate as thymidine. The A components isolated were, however, still impure after 4 chromatograms and it was impossible to identify the compounds on the basis of their ultraviolet spectra. The B deoxyribosidic material, however, was identified as deoxycytidine on the basis of its migration rate and spectrum. The final B fraction showed an absorption maximum at 279 to 280 mμ and a minimum at 243 mμ (*cf.* table 2). The optical-density ratios 280:260 and 250:260 were 1.81 and 0.58, respectively (*cf.* table 2). The amount of deoxycytidine found in the final B fraction by microbiological assay, from the cysteine-sulfuric acid reaction, and from the optical density at 280 mμ in acid was 67.6, 67, and 62 μg., respectively.

TABLE 3.—*Paper chromatography of hepatoma deoxyribosides**

Solvent	R_f values†	
	Hepatoma fraction A	Hepatoma fraction B
Butanol-H_2O	0. 67	0. 39
Butanol-H_2O-acetic acid		
Butanol-H_3BO_3	0. 35, 0. 47	0. 21
H_2O, *p*H 10	0. 61, 0. 75	0. 71
Isopropanol-H_2O-NH_4OH	0. 63, 0. 69	0. 52

*Hepatoma fractions A and B were chromatographed with the solvents listed above and applied consecutively, with reisolation of the deoxyribosidic compounds after each chromatogram. The 2 spots appearing in fraction A were separated and run individually on the rest of the chromatograms.

†*See* table 1.

In order to identify the other 2 hepatoma nucleoside components, the Dowex-1 system was used. The hepatoma extract at *p*H 7 was first passed through Dowex-1-formate and the column was washed with water until the optical density of the effluent at 260 mμ reached base-line values. In marked contrast to the results with liver, a large fraction of the total deoxyribosidic activity of the hepatoma extract was adsorbed on the column. In one experiment, 47 percent was so retained and in another, 41 percent. This indicated that a large proportion of the tumor deoxyribosidic compounds were nucleotides.[7] The effluent or nucleoside fraction was adjusted to *p*H 9.5 and rechromatographed on Dowex-1-formate (*cf.* text-fig. 1). Sixty-nine percent of the deoxynucleoside activity was recovered in the *p*H 9.5 eluate (deoxycytidine fraction), while the remainder appeared in the eluate at *p*H 8. The *p*H 8 eluate was then concentrated and chromatographed on paper, as shown in table 4. It will be noted that the R_f of the slower component in each chromatogram closely matched that of pure deoxyuridine, while the R_f of the faster component corresponded to that of pure thymidine. This identification was substantiated by the positions of the ultraviolet absorption maxima and minima (table 5). The amounts of thymidine and deoxyuridine actually isolated from 100 gm. of tumor were as follows:

Method of measurement	*Deoxyuridine* (*μg.*)	*Thymidine* (*μg.*)
Microbiological assay	22. 7	18. 6
Cysteine-sulfuric acid reaction	23. 9	18. 9
Calculation from optical density at absorption maximum	23. 2	18. 6

On the basis of the amount of microbiological activity recovered in the *p*H 9.5 eluate of the nucleoside fraction of the hepatoma and the fact that approximately equal amounts of deoxyuridine and thymidine were isolated, the concentrations of deoxycytidine, deoxyuridine, and thymidine in the hepatoma would appear to be in the ratio 5:1:1.

[7] Although experiments are in progress on the isolation of the nucleotide components, none of these compounds has yet been identified. Additional evidence that deoxynucleotides are present is furnished by the fact that over 40 percent of the deoxyribosidic activity of the hepatoma extracts was precipitated by barium acetate and ethanol.

TABLE 4.—*Paper chromatography of pH 8 eluate of hepatoma extracts* *

Solvent	R_f†			
	Tumor	Deoxycytidine	Deoxyuridine	Thymidine
Isopropanol-H_2O-NH_4OH	0. 50, 0. 60	0. 43	0. 50	0. 61
Butanol-H_3BO_3	0. 33, 0. 46	0. 18	0. 33	0. 43
Butanol-H_2O	0. 22, 0. 41	0. 17	0. 23	0. 40
Isopropanol-HCl-H_2O	0. 75, 0. 85	0. 54	0. 75	0. 84

*After the first chromatogram, the 2 tumor spots were chromatographed separately.
†*See* table 1.

TABLE 5.—*Spectral characteristics of tumor deoxyribosides* *

Material	Absorption maximum ($m\mu$)	Absorption minimum ($m\mu$)	Optical-density ratios†	
			$\frac{280}{260}$	$\frac{250}{260}$
Tumor deoxyriboside R_f=0. 75	262	230	0. 31	0. 75
Pure deoxyuridine	262	231	0. 34	0. 77
Tumor deoxyriboside R_f=0. 85	266	234	0. 63	0. 68
Pure thymidine	267	235	0. 67	0. 65

*Measurements were made in 0.02 *N* HCl after final chromatogram, table 4.
†*See* table 2.

Discussion

The results of the present study show clearly that the deoxyribosides occurring in rat liver and in rat hepatoma were qualitatively different. A single deoxyriboside, deoxycytidine, was isolated from the liver; in addition to this compound, thymidine and deoxyuridine were also isolated from the tumor. The fact that only pyrimidine deoxynucleosides have been detected in these tissues suggests that the compounds are not products of tissue autolysis but may represent precursors of DNA. Proof of this point must, however, await isotopic experiments involving the determination of the turnover of these compounds after administration of a DNA precursor. Hecht and Potter (*12*) have, in fact, done such experiments with rat liver after partial hepatectomy and have observed the rapid labeling of the acid-soluble deoxycytidine in this tissue. The fact that deoxycytidine, thymidine, and deoxyuridine are all found in the Novikoff hepatoma, a rapidly growing tumor, is also of interest because Reichard and his coworkers (*13,14*) found that all 3 compounds were readily and specifically incorporated into rat-liver DNA. Friedkin and his coworkers (*15–17*) have also observed the specific incorporation of thymidine and deoxyuridine into DNA thymidine in other tissues. The pyrimidine deoxynucleosides in the hepatoma may then represent a pool used for the synthesis of the DNA required by the constant growth of this tissue.

The large proportion of nucleotide-like deoxyribosidic material in the tumor is also of considerable interest. In the case of rat liver, less than 10 percent of the total deoxyribosidic material was present in this form (*11*), while in the hepatoma more than 40 percent appeared to be in the form of nucleotides. Since both tissues have approximately the same total amount of deoxyribosidic material, the concentration of deoxynucleotides in the tumor is at least 4 times as great as in normal rat liver. Similar compounds also account for a large proportion of the deoxyribosidic material in extracts obtained from the livers of rats after partial hepatectomy before regeneration of the livers occurs (*11*).

Although the hepatoma deoxynucleotides have not been identified, the type of compound present has been confined within certain limits by recent experiments with Dr. Richard L. Potter (*18*) and Dr. Robert L. Sinsheimer (unpublished). These studies have shown that the growth requirement of the organism used for the detection of the deoxyribosidic compounds cannot be satisfied by the di- or triphosphates of thymidine or deoxycytidine. On the other hand, the organism grows as well when supplemented with pure deoxydinucleotides as it does in the presence of thymidine. These results would seem to indicate that the hepatoma deoxynucleotides detected are either simple nucleotides or small polynucleotides.

The fact that deoxynucleoside di- and triphosphates could not be detected in our studies is of considerable importance, since Potter and Schlesinger (*19*) have isolated the di- and triphosphates of thymidine and deoxycytidine from the acid-soluble fraction of thymus glands and since the studies of Kornberg *et al.* (*20*) implicate these compounds in DNA synthesis. These findings pose the question whether such phosphorylated compounds might also be present in the acid-soluble fractions of the tissues studied here.

Summary

1) The deoxyribosides present in the acid-soluble fraction of normal rat liver and the Novikoff rat hepatoma were isolated by means of ion exchange and paper chromatography. The isolated compounds were identified from their migration rate on paper and by spectral, microbiological, and deoxyribose determinations.

2) The nucleoside deoxycytidine accounted for almost all of the deoxyribosidic compounds in normal rat liver.

3) The Novikoff hepatoma contained a markedly different spectrum of deoxyribosidic compounds. In addition to deoxycytidine, this tissue also contained deoxyuridine and thymidine. The 3 deoxyribosides appeared to be present in the ratio of 5:1:1 and accounted for 53 to 59 percent of the total acid-soluble deoxyribosidic material of the hepatoma. The remaining 41 to 47 percent of the deoxyribosidic compounds of the tumor exhibited properties suggestive of deoxynucleotides. These compounds have not yet been identified.

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Deoxyribosidic Compounds in Regenerating Liver [1]

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In a study of the occurrence, in animal tissues, of deoxyribosidic compounds, other than deoxyribonucleic acid (DNA), it was observed that the concentration of these compounds in mouse tumors was much greater than in most normal mouse tissues and that the types of deoxyribosidic compounds present in a transplantable rat hepatoma and in normal rat liver were qualitatively different (*1,2*). If the deoxyribosidic compounds present in these tissues were precursors of DNA, one would expect their concentration or turnover rate to increase in normal rat liver after partial hepatectomy. Hecht and Potter (*3*) have reported that deoxycytidine, the main soluble deoxyribosidic compound present in rat liver (*1*), is rapidly labeled with isotopes shortly after partial hepatectomy. In the present paper, the concentration of deoxyribosidic compounds in the liver after partial hepatectomy and some observations on the type of deoxyribosidic compound present in the liver after this operation are reported.

Methods

Male Sprague-Dawley rats, ranging in weight from 150 to 250 gm., were used in the experiments. In one group of animals the median and left lateral lobes of the liver were removed under sodium pentobarbital (Nembutal, Abbott) or ether anesthesia, according to the method of Higgins and Anderson (*4*). These lobes were frozen immediately in liquid nitrogen and kept at −20° C. for 1 to 7 days, when they were analyzed at the same time as the regenerating liver lobes for which they served as controls. Storage for these periods of time did not affect the total content of deoxyribosidic compounds significantly. At various time intervals after the operation, the animals were anesthetized and the blood vessels in their necks were severed. The regenerating livers were quickly excised and dropped into liquid nitrogen. Care was taken not to include any necrotic stump of liver that might have been present. At intervals after the operation another group of animals, subjected to simple laparot-

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omy, was also killed, and their livers were removed and frozen in liquid nitrogen.

The frozen tissue was weighed and homogenized [in glass tubes using Kel-F plastic pestles (*5*)] with a volume of 6 percent perchloric acid (PCA) equivalent to twice the weight of the tissue. After centrifuging at 18,000 r.p.m. and 0° C. (International rotor #290) for 30 minutes, the supernatant was brought to *p*H 7 with KOH. The neutralized extract was allowed to stand at 0° C. 18 to 24 hours before filtering off the insoluble $KClO_4$. Duplicate aliquots at several extract levels were then assayed for content of deoxyribosidic compounds with the organism *Lactobacillus acidophilus* R-26 (ATCC #11506) by the modified method (*1,2*) of Hoff-Jorgensen (*6*). This organism can utilize equally well any of the natural deoxynucleosides or deoxynucleotides for growth. Those aliquots of the tissue extracts producing growth falling within the linear portion of a standard thymidine growth-response curve were averaged to furnish the basis for calculations of the amount of deoxyribosidic compounds present in the original frozen tissue.

The deoxynucleotide content of tissue extracts was determined by passing 3 ml. of the extracts adjusted to *p*H 7.0 through Dowex-1-formate columns [5.0 × 0.9 cm., prepared as described by Hurlbert *et al.* (*7*)] followed by 15 ml. of water. The deoxynucleotides were adsorbed under these conditions and the eluate, containing the tissue deoxyribosides (*2*), was assayed microbiologically. The deoxynucleotide content was calculated as the difference between the content of deoxyribosidic compounds in the original extract and in the eluate.

Separation of the deoxyriboside fraction, obtained as in the previous paragraph, into a deoxycytidine fraction (*p*H 9.5 eluate) and a thymidine-deoxyuridine fraction (*p*H 8 eluate) was accomplished by the use of Dowex-1-formate columns, as described previously (*2*).

DNA was determined with the diphenylamine reagent on trichloroacetic acid extracts of a portion of the frozen tissue, following the method of Schneider (*8*) but omitting the phospholipide extraction.

The number of nuclei in the livers was determined essentially as described (*9*) for homogenates of these tissues in isotonic saline. Counts were made on at least 2 aliquots of each mixed suspension, and 2 separate homogenates were prepared for each tissue.

Results

In table 1, levels of deoxyribosidic compounds in the regenerating liver removed at intervals after partial hepatectomy are compared with levels in the original liver removed at the time of operation. The values for individual rats are listed in order, so that the concentration of these compounds in the regenerating liver can be compared directly with that present in the tissue from which it was derived. An examination of the table shows that the content of deoxyribosidic compounds had increased 60 percent 24 hours after partial hepatectomy. This high level of deoxyribosidic compounds continued through the 2nd day following partial

hepatectomy and at the end of 7 days was still greater than normal. Furthermore, since the concentration of deoxyribosidic compounds in the liver was greater in 3 of 4 rats examined only 12 hours after partial hepatectomy, the rapid production of these compounds was clearly indicated. The results in table 1 also show that, with the exception of one 12-hour rat liver, the level of deoxyribosidic compounds was greater in each regenerating liver than in the original liver removed at the time of the operation. Less extensive data on 2 strains of mice (table 2) suggest that an increase in concentration of deoxyribosidic compounds also occurs in mouse liver undergoing regeneration.

TABLE 1.—*Effect of partial hepatectomy on content of deoxyribosidic compounds in rat liver**

Time after partial hepatectomy (*hours*)	Content of deoxyribosidic compounds†		
	Excised liver‡	Regenerating liver	Regenerating / Excised
12	**8.2** (9.9, 8.1, 7.1, 7.8)	**9.4** (9.2, 9.9, 9.0, 9.6)	**1.14**
24	**12.1** (15.4, 9.5, 11.2, 11.1, 13.6, 12.1)	**19.4** (21.2, 15.4, 17.9, 18.3, 23.8, 20.0)	**1.60**
48	**10.0** (10.2, 13.3, 9.3, 6.5, 10.6)	**17.1** (16.4, 20.0, 17.2, 14.2, 17.8)	**1.71**
72	**11.5** (9.4, 14.1, 16.7, 8.4, 11.2, 8.9, 11.7)	**17.7** (15.4, 18.3, 19.7, 17.2, 18.2, 20.6, 14.5)	**1.54**
96	**10.8** (11.4, 8.1, 9.1, 14.6, 10.7)	**17.2** (14.4, 12.9, 20.0, 25.6, 13.0)	**1.59**
168	**12.4** (15.9, 11.0, 10.6, 12.1)	**16.2** (20.0, 13.1, 14.1, 17.6)	**1.31**

*Numbers in boldface = average values; numbers in parentheses = values for individual rats.
†Determined microbiologically as described in the text and reported as μg. of thymidine per gm. of frozen tissue.
‡Liver lobes removed at zero time, *i.e.*, at the time of partial hepatectomy.

TABLE 2.—*Effect of partial hepatectomy on content of deoxyribosidic compounds in mouse liver*

Mouse strain	Time after operation (*hours*)	No. of livers pooled	Content of deoxyribosidic compounds*	
			Excised liver†	Regenerating liver
C3H	24	4	1.1	1.6
DBA	24	3	1.1	1.1
DBA	48	4	1.1	1.8
DBA	48	5	1.2	2.4

*Determined microbiologically as described in the text and reported as μg. of thymidine per gm. of frozen tissue.
†Liver lobes removed at zero time, *i.e.*, at the time of partial hepatectomy.

In order to test whether the operative procedures prior to removal of the liver might lead to an increased concentration of deoxyribosidic compounds, a number of rats were laparotomized and their livers were assayed at intervals after the operation. The results (table 3) show that the level of these compounds in the liver, after laparotomy, fell within the range of the control excised livers (table 1). Furthermore, the mean for the laparotomized rats was 12.0 μg. of thymidine per gm. of liver as compared with 11.3 μg. for the corresponding excised livers in table 1.

When the normal rat-liver extracts were passed through Dowex-1 columns, an average of 92 percent of the deoxyribosidic compounds in the excised tissue extracts was recovered in the eluate from the columns, whereas only 67 and 72 percent were found in similar eluates from extracts of 24-hour and 48-hour regenerating liver, respectively (table 4). These findings suggest that deoxynucleotides are present in much greater concentration in regenerating liver. Moreover, the increased concentration of deoxyribosidic compounds in regenerating liver would appear to be accounted for largely by the increase in the deoxynucleotide fraction.

TABLE 3.—*Content of deoxyribosidic compounds in livers of laparotomized rats*

Time after laparotomy (*hours*)	Content of deoxyribosidic compounds*
24	12. 2
48	13. 8, 13. 6, 9. 1, 7. 9
72	10. 2, 9. 7
96	10. 2, 14. 1
168	11. 1, 10. 1, 12. 7, 15. 1

*Determined microbiologically as described in the text and reported as μg. of thymidine per gm. of frozen tissue.

TABLE 4.—*Effect of partial hepatectomy on retention of liver deoxyribosidic compounds by Dowex-1 columns*

Time after operation (*hours*)	No. of experiments	Percent of deoxyribosidic compounds recovered in eluates from Dowex-1 columns
0 (excised liver lobes)	6	92 (87–100)
24	7	67 (48–77)
48	5	72 (62–78)

Since deoxynucleotide-like compounds also comprised a large proportion of the deoxyribosidic compounds of the hepatoma (*2*) and since the tumor also contained deoxynucleosides besides the deoxycytidine found in normal rat liver, it was of interest to determine whether the deoxynucleoside pattern in the liver was altered after partial hepatectomy. To this end, the trichloroacetic acid (TCA) extract from 30 gm. of liver removed from rats 48 hours after partial hepatectomy was freed of

TCA with ether, neutralized to *p*H 7, and applied to a Dowex-1-formate column. The column was then washed with distilled water until the optical density at 260 mμ had returned to base-line levels. The eluate, containing any deoxynucleosides present in the original extract (*2*), was brought to *p*H 9.5 with NH_4OH and passed through Dowex-1-formate previously equilibrated at *p*H 9.5. After washing the column with *p*H 9.5 buffer until the optical density of the effluent returned to base-line levels, the column was washed exhaustively with *p*H 8 buffer and water. Microbiological assay showed that the entire microbiological activity was present in the *p*H 9.5 or deoxycytidine fraction. Thymidine and deoxyuridine thus appeared to be absent from these livers in measurable amounts. In other experiments, which need not be described in detail here, the nucleoside present in the *p*H 9.5 eluate of regenerating liver has been isolated and identified as deoxycytidine.

In view of the possible significance of deoxyribosidic compounds as precursors of DNA and of the long-standing interest in DNA in growing tissues, the DNA content of regenerating liver was reinvestigated. No difference was observed in the number of nuclei in tissue freshly excised and homogenized or tissue frozen in liquid nitrogen, weighed, and allowed to thaw before homogenization. Counts obtained from the same tissue gave values of 207×10^6 and 210×10^6 nuclei per gm. of fresh and frozen liver, respectively.

Table 5 summarizes data obtained from those experiments relating DNA with cell number. In agreement with the results of previous investigators [summarized (*10*)], the number of nuclei per gm. of liver decreased following partial hepatectomy, while the DNA content of the liver remained about the same. The amount of DNA per nucleus was thus greater in the regenerating livers than in the controls. When referred to the number of nuclei in the tissue, the percentage increase in deoxyribosidic compounds per nucleus after partial hepatectomy was even greater than the increase observed per gm. of liver (table 5).

Discussion

The results presented in this paper show clearly that the concentration of deoxyribosidic compounds in liver increased markedly after surgical removal of two thirds of the liver. This increase in concentration was already nearly maximal 24 hours after the operation. Since mitosis in the liver cells begins subsequent to this time (*11*), the present results are in harmony with the thesis that the tissue deoxyribosidic compounds are precursors of DNA.

It is of considerable interest that the types of deoxyribosidic compounds in the liver after partial hepatectomy were different from those in normal liver. In the regenerating liver, as much as 33 percent of the total deoxyribosidic compounds appeared to be nucleotides, while in normal liver over 90 percent of these compounds were accounted for as the nucleoside deoxycytidine. The nucleotides accounted for a major part of the increased concentration of deoxyribosidic compounds in the regenerating liver

TABLE 5.—*Effect of partial hepatectomy on DNA content and number of nuclei in rat liver—each value average is of determinations on 3 rats*

Time after operation (*hours*)	DNA content (*mg. per gm. liver*)		Nuclei ($\times 10^{-6}$ *per gm. liver*)		DNA per nucleus (*picograms*)		Content of deoxyribosidic compounds per nucleus*		
	Excised†	Regenerating	Excised†	Regenerating	Excised†	Regenerating	Excised†	Regenerating	Regenerating / Excised
24	2. 39	2. 38	195	160	12. 2	14. 8	6. 2	12. 1	1. 95
48	2. 44	2. 56	232	172	10. 5	14. 9	4. 3	9. 9	2. 30
72	2. 68	2. 85	220	207	12. 7	13. 8	5. 2	8. 0	1. 54

*Expressed as μg. thymidine $\times 10^7$ per nucleus and calculated from the results in table 2.
†Liver lobes removed at zero time, *i.e.*, at the time of partial hepatectomy.

and would seem to be of special importance in growing tissues, since they have also been found in large amounts in a rapidly growing transplantable hepatoma (*2*). The identification of the deoxynucleotides consequently assumes great importance and is now being actively pursued in this laboratory.

Summary

1) The concentration of deoxyribosidic compounds was determined microbiologically in cold perchloric acid extracts of rat and mouse liver at various intervals after laparotomy and partial hepatectomy. The concentration of these compounds in the liver increased more than 60 percent following partial hepatectomy but did not change after laparotomy.

2) The increased concentration of deoxyribosidic compounds in the regenerating liver was accounted for in large part by deoxynucleotide-like compounds, which comprised as much as 33 percent of the total. In normal rat liver, deoxynucleotides accounted for only 8 percent of the total deoxyribosidic compounds.

3) The number of nuclei in the liver and the DNA content of the liver were also determined after partial hepatectomy. The increased amount of DNA per nucleus observed during regeneration was associated with a decrease in the number of nuclei per gm. of liver. The amount of deoxyribosidic compounds per liver nucleus increased more than 95 percent after partial hepatectomy.

4) Since the increase in the concentration of deoxyribosidic compounds in the liver occurred before the cell divisions leading to restoration of the original liver, the results were considered to be in harmony with the thesis that the deoxyribosidic compounds represented precursors of DNA.

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A Transplantable Mast-Cell Neoplasm in the Mouse [1]

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One value of neoplasms is the information they may furnish regarding the function and behavior of the normal cell or organ counterpart. This is especially true if the neoplasm can be transplanted and studied under controlled conditions. Mast-cell neoplasms are rare in mice (*1*), and we know of only one that was previously transplanted (*2*). The transplantable mast-cell neoplasm to be described has now been carried through 12 generations and has been converted to ascites form. This has given us an opportunity to study its morphology, its behavior under a variety of conditions, and its effect on the host.

Observations

Original Tumor

The tumor arose in a strain DBAf/2 male mouse that had been orchiectomized at 5 months of age. The skin was painted with 0.2 percent methylcholanthrene in ether 3 times weekly through an exposure time of approximately 7 weeks. To minimize the development of skin cancer, the carcinogen was applied to 8 different areas; no area was ever painted twice in succession. A total of 20 applications was made; thus, 4 areas received 3 applications; the other 4 received only 2. At 16 months of age the animal was found to have a small ulcerated tumor on the back and a large mass over the sternum. The sternal mass was adherent to the skin and underlying structures and was ulcerated at one point. The abdomen was enlarged. Otherwise, the animal appeared to be in good health. It was killed with ether. At autopsy the mass overlying the sternum measured 1.5 $\times$ 0.5 cm. It was composed of very soft tissue, which was whiter and more opaque than the tissue of lymphocytic neoplasms that had been encountered previously in mice of this group receiving similar treatment. The tissue forming the margins of the ulcer was canary-yellow in color. The spleen and liver were greatly enlarged, mottled, and pale. The lymph nodes were not enlarged, but

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some showed areas of white, opaque tissue. Because of the unusual appearance, tissues from the mass overlying the sternum and from the spleen were transplanted into 3 other DBAf/2 mice.

The tissue was routinely fixed in Zenker-formol. Histologic sections stained with hematoxylin and eosin revealed that the smaller skin mass from the dorsal area was a squamous-cell carcinoma. The sternal mass consisted entirely of faintly eosinophilic cells with round, oval, crescentic, or ring-shaped nuclei and relatively abundant cytoplasm. Scattered nests of similar cells appeared in the lymph nodes, and there was a heavy infiltration of such cells in the spleen and liver (fig. 1). Other organs appeared normal. In sections stained with toluidine blue (0.5% aqueous solution at *p*H 4) the neoplastic cells showed great variation in intensity of staining. One area from a lymph node showed deep coloration of one group of cells, while cells in a nearby area were more faintly stained (fig. 2). This difference was caused by variations in size and number of the metachromatic granules within the neoplastic cells. Most of the cells in the sternal tumor were poorly stained (fig. 3), and the granules were usually sparse and delicate, requiring high magnification to discern them. It was noted that more intense metachromasia was found in necrotic than in viable areas of the sternal mass. Granules in cells adjacent to areas of necrosis were aggregated and could be seen more distinctly. After treatment with the periodic acid-Schiff method, the cytoplasm of the cells was colored a deep pink. The granules could not be seen distinctly. Wilder's reticulum method did not reveal any fibers in close association with the tumor cells.

Transplant Behavior

The first transplants were made to 3 DBAf/2 male mice 3 months of age. A small bit of tumor tissue was transferred with a trocar to the subcutaneous tissue in the flank. The transplant grew in all 3 mice; indefinitely outlined masses were palpated at the site of transfer in about 10 days. Transplantation to the next generation was made from 2 of these mice after periods of 2 and 3 weeks, respectively, when the subcutaneous masses were less than 1 cm. in diameter. Transfers are now being made every 4 to 5 weeks, and the tumor is in the 12th generation. The solid-tumor line has grown in nearly all DBAf/2 animals, and (BALB/c $\times$ DBAf/2)F_1 hybrids. It has failed to grow in C3Hf mice or in (C57L $\times$ A)F_1 hybrids. The transplanted tumors have reproduced the morphologic features of the original tumor, except that the cells of the transplants appeared to be more uniform and there was less variation in the degree of granulation, which was generally sparse and delicate. No masses of intensely granulated cells closely resembling normal mast cells have ever been found in the transplanted tumors. This observation suggests that it was the less-differentiated cells from the original tumor that grew in the new hosts. The subcutaneous transplants infiltrated adjacent skin and muscle (fig. 4), and secondary deposits in the spleen and liver were frequent.

At the third transfer, an ascites line was developed following the intraperitoneal injection of a tumor brei. After 2 weeks, the abdomen was swollen and about 1 ml. of ascites fluid could be removed. The fluid contained numerous neoplastic mast cells and only a few erythrocytes. Loose tissue of the mesentery, spleen, and liver contained small masses of white, opaque tissue. Passage of the ascites form has been made every 2 weeks by means of the free fluid in the peritoneal cavity. Smears from the fluid gave an excellent picture of the neoplastic mast cell (figs. 5, 6, 7). The total accumulation of ascites-tumor cells in the peritoneal cavity following inoculation of approximately 1×10^6 cells is on the average 250×10^6 cells in 11 to 14 days. The neoplasm has consistently been a nonhemorrhagic ascites tumor. One of the most striking characteristics is the small amount of ascites fluid usually found, that is, the cells are very densely populated in a small fluid volume. The approximate number of cells required for successful transfers can now be estimated, and the results of injecting different numbers of cells was observed in one series (table 1). It was found that the time of death was rather closely correlated with the number of cells injected. The interval ranged from 12 to 30 days. When a dose of 10^6 cells was given, the majority of the deaths were on the 15th and 16th days. This standardization of the cell-dose response has made it possible to evaluate other factors related to the tumor.

TABLE 1.—*Time of death, in days, following injection of measured number of cells**

Day of death after transfer at following cell dose:		
1.5×10^6	1.5×10^4	1.5×10^2
12	20	23
14	20	24
15	20	25
15	20	25
16	21	25
16	21	25
16	24	26
18	30	29
Average 15. 2	22. 0	25. 2

*Generation 6; ascites line; 8 animals in each group.

Effect of Tumor on Host

In order to determine whether the mast-cell tumors had any special effect on the host, the ascites fluid from a single mouse was mixed with Locke's solution, and 2 ml. aliquots of this cell suspension were injected into the flank of 10 male and 10 female (BALB/c $\times$ DBAf/2)F_1 hybrids, 2 months old. This method insured a uniform dose to all the experimental mice. Five male and 5 female mice of the same age from the same group of hybrids were set aside as uninoculated controls. At the end of 22 days, 1 male and 1 female of the tumor-bearing group of mice

died. Within the next 2 days the remaining tumor-bearing and all untreated mice were killed and autopsies were performed. All the injected mice had large subcutaneous tumors, many of which had ulcerated, so that some of the tumor tissue had been extruded. Each mouse was first weighed, then killed with ether, and the subcutaneous tumor dissected free and weighed. The spleens and livers from all the tumor-bearing and control mice were also weighed (table 2).

TABLE 2.—*Comparative weights of male and female tumor-bearing mice and controls**

Mice	No.	Animal weight (*gm.*)	Tumor weight (*gm.*)	Spleen weight (*gm.*)	Liver weight (*gm.*)
Males with tumor	9	**22.1** *19–24.5*	**1.34** *0.47–2.28*	**0.33** *0.23–0.45*	**1.81** *1.39–2.76*
Control males	5	**23.5** *21.4–25*	— —	**0.15** *0.10–0.16*	**1.69** *1.50–1.73*
Females with tumor	9	**22.5** *20.5–24*	**1.54** *0.59–2.83*	**0.25** *0.21–0.33*	**1.43** *1.32–1.67*
Control females	5	**22.5** *21–23.5*	— —	**0.10** *0.09–0.12*	**1.47** *1.42–1.58*

*Figures in bold-face type=average; figures in italics=range.

The differences in weight were insignificant. The local tumors were slightly larger in the females and the spleens and livers were smaller, indicating in general that these viscera contained smaller tumor deposits in the females, although individual cases varied greatly. The small size of some of the local tumors was probably the result of loss of tissue by ulceration. Unexpected death among tumor-bearing animals was a frequent occurrence, and signs of impending death usually developed rapidly.

Autopsies were carried out under a dissecting microscope at 10× magnification. Numerous pieces of tissue were fixed including eye, brain, Harderian gland, salivary glands, all endocrine organs, and all reproductive organs. Sections were stained with hematoxylin and eosin and toluidine blue. A comparative study was made of tumor-bearing and control mice of both sexes. No difference was found between the sexes, with the possible exception of more widespread dissemination of tumor cells in the males. No significant alteration from the normal was found in the organs of the tumor-bearing mice, except for infiltration by tumor cells. Secondary deposits of tumor were found in the liver and spleen and occasionally in the mesentery, lymph nodes, and lung. Tumor cells were absent from the peripheral blood. Amyloidosis of the spleen was found in 3 male and 2 female tumor-bearing mice and atrophy of the thymus in 2 males, but these findings are frequent in mice bearing any large necrotic tumor.

Morphology of the Ascites-Tumor Cells

Smears from the ascitic fluid stained with Wright's stain gave an excellent opportunity to observe the morphology of the neoplastic cells. The

form of the nucleus and the disposition and size of the granules could be seen clearly (figs. 5, 6, 7). Many of the nuclei were ring- or doughnut-shaped, like the nucleus of the granulocyte in the mouse. The doughnut-shaped nucleus could also be seen in paraffin sections (fig. 8). Smears from the ascitic fluid showed great variation in the size of the granules in different cells, yet all the granules in a single cell tended to be of the same size. The cytoplasm of less-differentiated cells with large, round nuclei occupying most of the volume of the cell usually contained small delicate granules. The granules were not evenly distributed in many of the cells but appeared to be aggregated into a small mass.

Histology of the Transplanted Solid Tumors

The cells were remarkably uniform in size, with the variations in nuclear shape already described for the ascites form. The metachromatic granules were generally sparse and delicate, and high magnification was required to see them. Mitotic figures were frequent, and granules were present in cells in mitosis. The granules appeared aggregated and were most clearly seen in cells adjacent to areas of necrosis. Necrosis usually affected only small areas, even of large tumors. The normal tissue bordering neoplastic tissue showed no inflammatory reaction or any other change, except mechanical compression by tumor tissue. Metachromasia was more intense in necrotic areas than in areas composed of viable cells.

One observation may be significant. A peculiar canary-yellow color was observed in necrotic areas of the original tumor, and this has been observed regularly in the transplanted tumors. When tumor tissue with such yellow areas was cleared in glycerine and examined at high magnification with transmitted light, numerous yellow rhomboidal crystals were found (fig. 9). Such crystals are commonly seen in reticulum-cell sarcomas in mice (*3*) and have been called hematoidin crystals, following the designation used by Virchow for similar structures in human tissues (*4*). The crystals were birefringent under polarized light and disappeared during paraffin embedding. In addition to the crystals, tissue from the transplanted mast-cell tumors usually contained numerous bright-yellow birefringent globules (figs. 9, 10, 11), which remained in the tissue after embedding and staining (fig. 12). Often a large globule contained numerous crystals (fig. 11). The globules did not stain for iron with the Prussian-blue reaction and no iron particles could be identified after microincineration. Fat stains were difficult to interpret, because the globules were found in necrotic areas where fat was abundant and were mingled with fat-positive debris and macrophages with ingested fat. With oil red O in frozen sections, some yellow globules and crystals were found that lacked any red color. Others appeared to be faintly colored by the dye. Similar results with the globules appeared after Sudan-black treatment of paraffin sections, but most of the globules retained the clear yellow color. The globules were negative with an acid-fast stain.

Findings in other mice similarly treated.—A number of other mice of the same strain have been painted with methylcholanthrene, according to

the same schedule as that followed in the mouse which developed the transplantable mast-cell neoplasm. Many of these mice have not yet come to autopsy. So far, lymphocytic neoplasms have been frequent. Several mice have shown microscopic accumulations of mast cells in lymph nodes. In 1 mouse recently examined, a white, opaque area was seen in an inguinal node. Impression smears of this tissue stained with toluidine blue showed numerous mast cells. Transplantation of the small focus was made to 2 mice, but no growth has developed after 12 weeks. This same mouse had a lymphocytic neoplasm in the thymus.

Discussion

The successful transplantation of a mast-cell neoplasm is evidence that the mast cell in the mouse is an independent cell series capable of undergoing a malignant change. After the malignant change, the cell still maintained its distinguishing feature, which is the formation of metachromatic granules. The number of these granules per cell was greatly reduced when compared with the normal mast cell.

It is probable that this neoplasm was induced. Painting with carcinogenic hydrocarbons has been reported to cause an increase in the number of mast cells in subcutaneous tissue, and it was suggested that such proliferation of mast cells was a reaction against the neoplastic change developing in the epidermal tissue (*5*). It seems more reasonable, however, to suggest that the mast cells are themselves responding to the carcinogen and undergoing a neoplastic alteration. The greater susceptibility of other tissues to the carcinogen and the more rapid growth of squamous-cell carcinomas and subcutaneous fibrosarcomas may be a reason that fully developed, independent mast-cell neoplasms are infrequently obtained. Mast-cell neoplasms have been found in untreated mice, but these spontaneous neoplasms are usually small, the cells are well differentiated, mitoses are rare, and the growth rate is slow (*1*).

Morphologic study of mice bearing large transplanted mast-cell tumors failed to reveal any alteration that might indicate any special effect of the mast cells on the host. Analysis of tissue having numerous normal mast cells (*6*), and of mast-cell tumors of dogs (*7*), has shown a high content of heparin. A recent report (*8*) stated that normal mast cells and mast-cell tumors from dogs are rich in histamine. The present mast-cell tumor of the mouse contained a very large amount of histamine and serotonin. Reports of this finding will be published elsewhere.[3] As an explanation for the apparent inertness of the mast cell, it has been proposed that it is only when the granules are released that its function is exhibited. However, Devitt and coworkers (*9*) have demonstrated that the finding of free granules in histologic sections may be the result of artifacts. The mast-cell tumor in the mouse has shown little evidence of "release of granules" until after the tissue undergoes necrosis. It must be remembered, also, that the neoplastic counterpart of a cell often fails to show the functional activity of the normal cell, possibly because of failure of chemical as well

[3] Sjoerdsma, A., and coworkers.

as morphologic differentiation. For this reason the neoplasm may not necessarily be an indicator of normal cell function.

The morphology of the nucleus as seen in paraffin sections of solid tumors and in smears from ascites tumors was puzzling. The nucleus was often ring-shaped or doughnut-shaped and resembled the nucleus of the granulocytic series of cells in the mouse. This type of nucleus has not been recognized in the normal tissue mast cell, and the blood basophil is not found in mice. We have recently observed that some reticulum-cell sarcomas in an ascites form in the mouse have ring-shaped nuclei (*10*).

The consistent observation of hematoidin crystals and yellow globules in necrotic areas of the transplanted subcutaneous tumors at sites where hemorrhage almost certainly occurred suggests that the mast cell may have a function concerned with the formation of bile pigment from hemoglobin. No viable cells could be discerned in these necrotic areas. Similar crystals have been found regularly in reticulum-cell sarcomas in the mouse. Since the formation of bile pigment is considered to be a function of the reticulum cell, the finding of crystals in neoplasms composed of such cells is understandable. The crystals may also be found occasionally around the margin of other tumors, where normal reticulum cells are present, but are not found within the substance of fibrosarcomas, lymphosarcomas, or mammary and epidermoid carcinomas. Muir and Niven (*11*) reported the development of hematoidin crystals and yellow globules after the injection of whole bood into the subcutaneous tissue of mice and rats. It was their opinion that the histiocytes or reticulum cells were responsible for the conversion of hemoglobin and that the crystals developed intracellularly. In our experience, the crystals have been found only in necrotic areas and are nearly always extracellular. The presence of the crystals in both reticulum-cell sarcomas and the mast-cell neoplasm in the mouse suggests a relationship between the 2 types of cell. It is evident, however, that a particular set of conditions, including necrosis and hemorrhage, is required before the conversion of red-cell debris to bile pigment can occur. We did not search for crystals in the fresh tissue from the original tumor, and no globules could be found in the microscopic sections. An examination was made of mast-cell lesions in other species. A study of histologic sections of 10 lesions of urticaria pigmentosa, in which mast cells were abundant, and of 15 mast-cell tumors of the dog revealed no globules. Histologic sections are unsatisfactory, however, because the crystals are not preserved and necrotic areas are avoided when taking tissues for section. Fresh tissue from a dog with a metastasizing mast-cell neoplasm was obtained at autopsy. This failed to reveal crystals or globules, although necrotic, hemorrhagic areas were present and were selected for examination. This failure may be connected with a species difference, or the particular tumor may have lacked the character necessary for pigment formation. Muir and Niven remarked that yellow patches in which an abundant formation of hematoidin took place were not uncommonly found at the margin of adipose tissue, and often actually in its substance. An association with fat was also observed

in the formation of hematoidin crystals in the mast-cell neoplasms, and the presence of fat may also be a necessary condition for hematoidin formation.

In one previous report of a mast-cell lesion, yellow-pigment formation was noted. Sabrazès and Lafon (*12*) described a "granulome" composed of mast cells and eosinophils in a horse. The eosinophils formed anastomosing bands that delimited and separated islands of mast cells. A fistulous tract led from necrotic areas in the lesion to the skin surface. The exudate from the fistula was yellow, and small yellow masses were found in the tissue forming the wall. On microscopic examination, hemorrhagic foci were described. Within these were numerous large "hématomacrophages" loaded with yellow pigment. Hematoidin crystals were found in necrotic areas. The "détritus hématiques" did not give a strong Prussian-blue reaction, and only traces of iron pigment were found. The similarity of these observations to the yellow color and hematoidin crystals found in the mast-cell tumor in the mouse demands consideration, despite our failure to find any yellow pigment in human and canine lesions. It is hoped that additional transplantable mast-cell neoplasms in the mouse may be obtained, and that this pigment formation can be investigated further.

Summary

A transplantable mast-cell neoplasm in the mouse is described. The original tumor arose in a strain DBAf/2 mouse painted repeatedly with methylcholanthrene. The tumor has been carried in both solid and ascites forms.

Notable features of the tumor were:

1) Failure to show any special effect on the normal tissues and organs of tumor-bearing mice and failure to show any difference in behavior when grown in male or female hosts.

2) Excellent exhibition of cytologic details in neoplasms grown in the ascites form. A ring-shaped nucleus was clearly demonstrated in many of the neoplastic cells. The granules from different cells varied in size and number and were often aggregated within a small area in the cytoplasm.

3) Hematoidin crystals and unusual birefringent yellow globules in necrotic areas of the tumor. This may indicate that the mast cell has a function related to the formation of bile pigments from hemoglobin.

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PLATE 38

FIGURE 1.—Original mouse. Massive infiltration of liver by neoplastic mast cells. Strands of liver cells are compressed and atrophied. Mast-cell granules are not discernible with this stain. Hematoxylin and eosin. × 260

FIGURE 2.—Original mouse. Lymph node, showing masses of neoplastic mast cells with varying degrees of granulation. In *lower left*, cells contain numerous granules, so that nucleus is obscured when toluidine-blue stain is used. In *upper right*, cells contain fewer granules. Some of cells in *center* contain only a few delicate granules. Toluidine blue. × 260

FIGURE 3.—Original mouse. Section from large mass overlying the sternum. Majority of cells were only faintly stained, and very high magnification was required to see the sparse, delicate granules. Granules are more clearly seen in necrotic cells in *center*. Toluidine blue. × 610

FIGURE 4.—Subcutaneous transplanted tumor. Neoplastic cells in solid sheets are infiltrating skeletal-muscle fibers. Hematoxylin and eosin. × 260

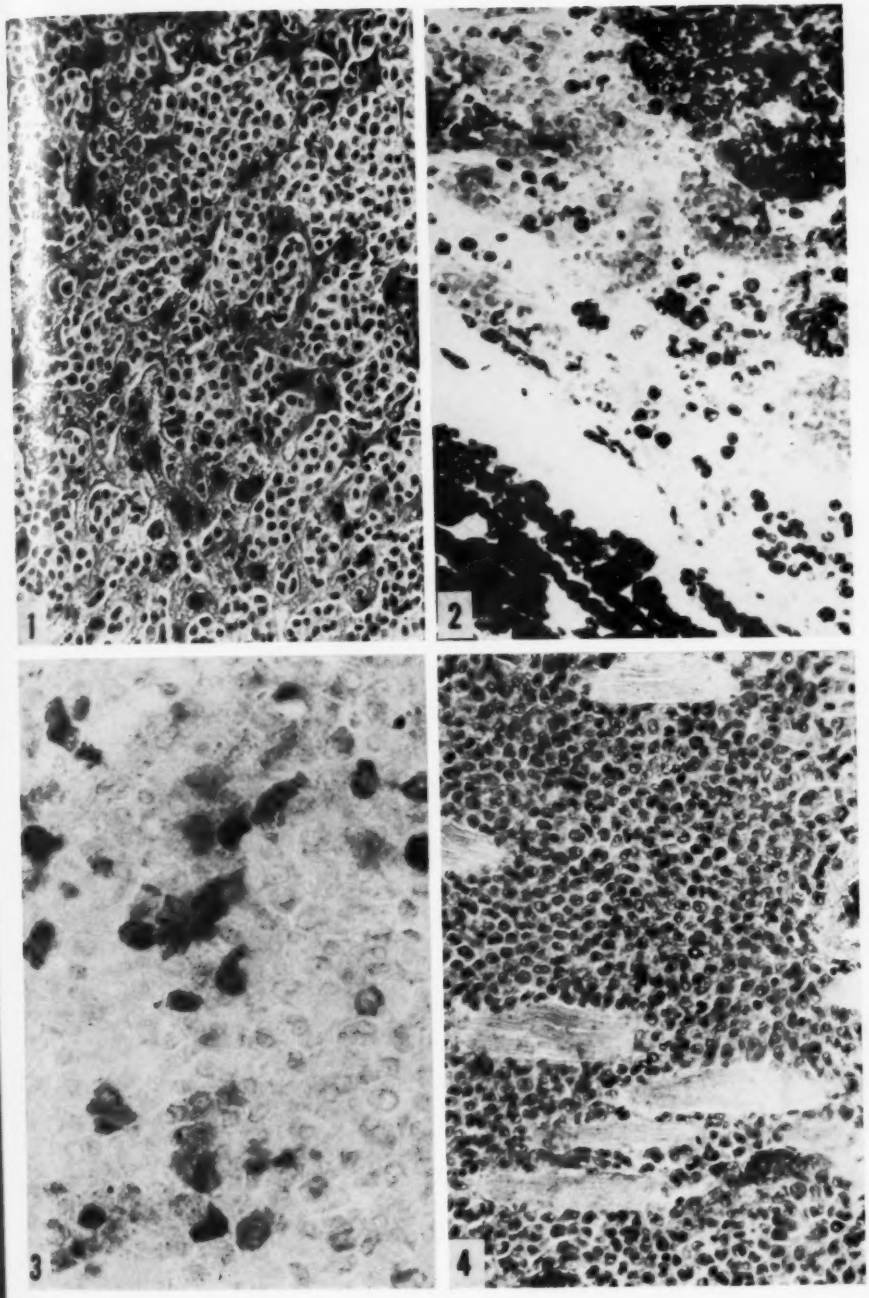

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PLATE 39

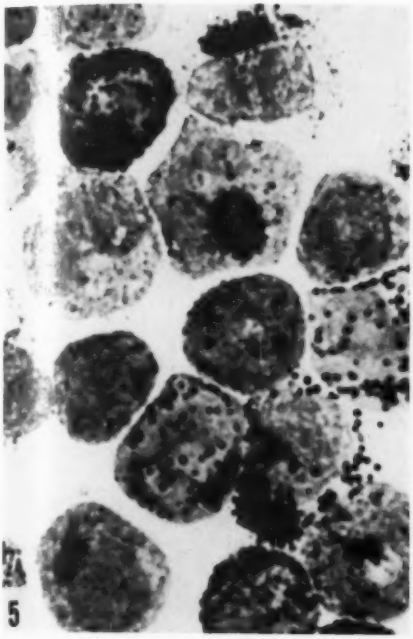
Figures 5, 6, and 7 show smears from ascites tumors. Variation in size and number of granules is shown. Wright's stain. × 1200

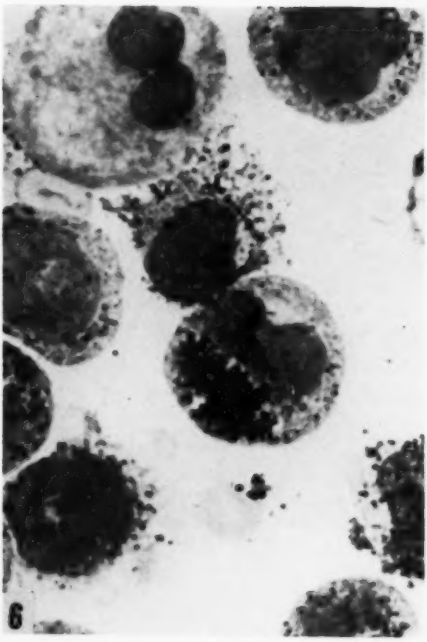
FIGURE 5.—Heavily granulated cell in *upper left* is probably a normal mast cell which may be present in ascitic fluid. Two cells in *upper center* show aggregation of granules.

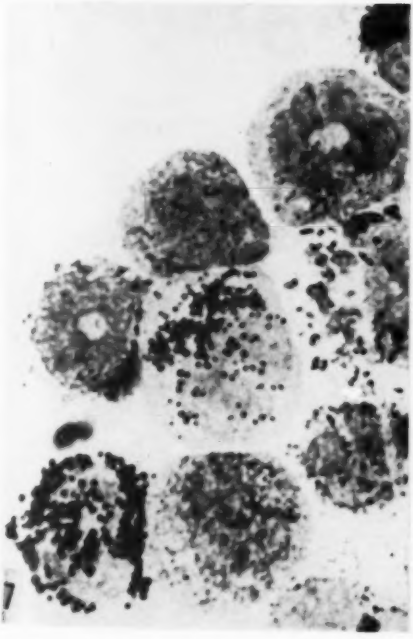
FIGURE 6.—A binucleated histiocyte, normally present in ascitic fluid, is shown in *upper left*. Mast cell near *center* contains a bilobed nucleus.

FIGURE 7.—Doughnut- or ring-shaped nuclei are shown. Cell in *center* contains large, distinct granules. In cell *above*, granules are delicate and indistinct.

FIGURE 8.—Microscopic section showing types of nuclei. A ring or doughnut form appears in *center*. Other nuclei are round, oval, indented, crescentic, or lobulated. Hematoxylin and eosin. × 880







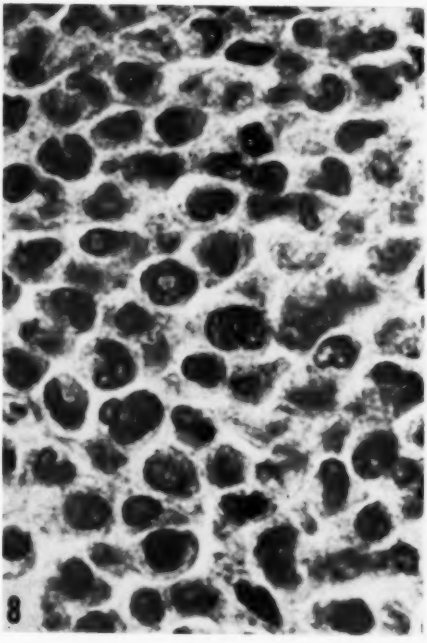


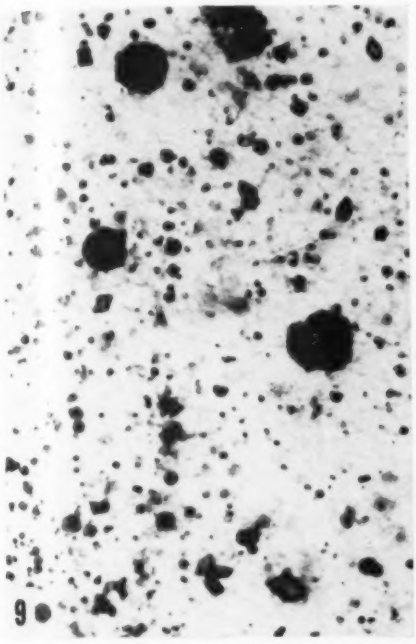
PLATE 40

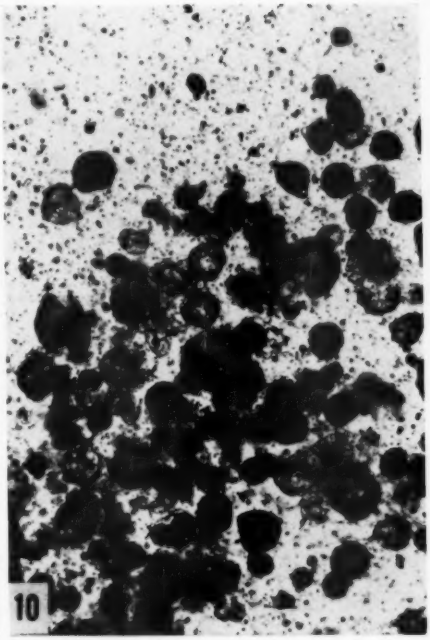
FIGURE 9.—A glycerine-cleared, unstained preparation of yellow tissue taken from necrotic area of transplanted tumor. A number of rhomboid crystals and several globules, which are bright yellow, can be seen. × 440

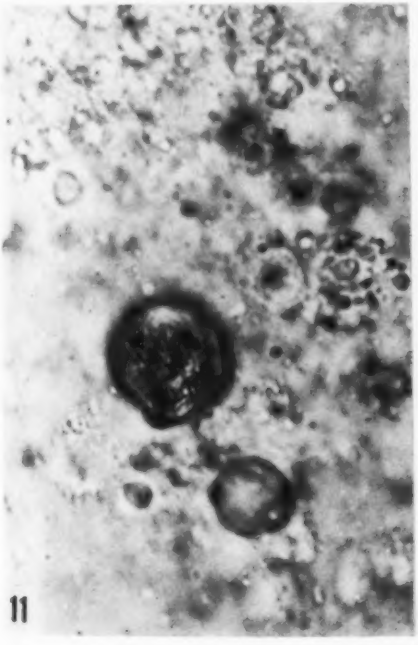
FIGURE 10.—Similar preparation showing an aggregation of yellow globules. A few crystals are also present. × 200

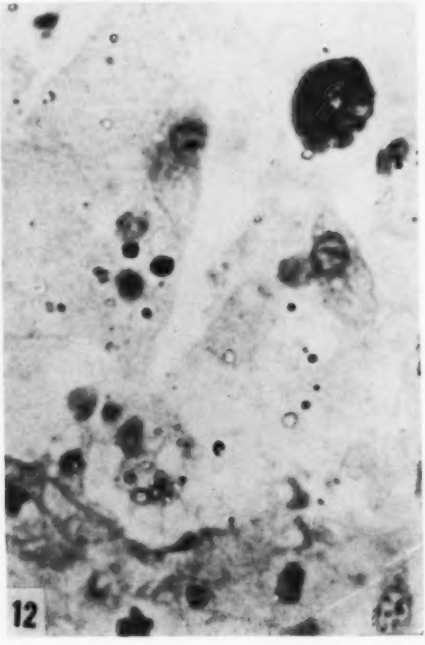
FIGURE 11.—A yellow globule containing a number of rhomboid crystals. In *lower right*, a smaller, more transparent yellow globule. × 915

FIGURE 12.—Microscopic section from a necrotic area in a transplanted tumor, showing a large yellow globule in *upper right*. The small, dark masses in *center* are also bright yellow. Any crystals would have been removed by paraffin embedding. × 880









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